ASSESSMENT OF THE ANTI-OBESITY EFFICACY AND MECHANISM OF A TRADITIONAL CHINESE HERBAL FORMULA

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The thesis is acceptable in form and content and a satisfactory knowledge of the field covered by the thesis was demonstrated by the candidate through an oral examination held on 22 March 2013.

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ABSTRACT

Obesity has become an increasingly common health condition over the last several decades. It has been estimated that this largely preventable disease affects over one billion people worldwide and that its prevalence will have doubled by the year 2030. Currently, obesity is the leading cause of preventable deaths worldwide and is predicted to become the biggest public health problem of the century. For the treatment and management of obesity, traditional Chinese medicine (TCM) has become an increasingly attractive form of alternative/complementary medicine. This trend is also occurring in the face of a weight-loss product market largely typified by mediocre long-term efficacy and safety concerns, and an increasing global desire to seek out and develop safe and efficacious products from natural sources. This thesis aimed to assess the anti-obesity efficacy and mechanism of the traditional Chinese herbal formula, Liuwei Dihuang (LWDH), using both *in vivo* and *in vitro* approaches.

An *in vivo* study using obese-prone rats treated by twice-daily oral gavage with LWDH showed the body weight- and food intake-lowering efficacy of the product, which also favourably affected body fat composition. Subsequent biochemical analyses revealed the lipid-lowering, as well as leptin- and insulin-sensitizing efficacy of LWDH, in addition to its beneficial effects on biomarkers of inflammation and oxidative stress and adiponectin production. Liver function testing, assessed by measuring liver enzymes levels in the serum, revealed no adverse side effects of LWDH in rats under the experimental conditions of this thesis.

The potential anti-obesity mechanisms of action subsequently investigated included regulation of appetite and/or fatty acid metabolism. Using collected ileal and hypothalamic tissues of LWDH-treated obese rats, and cell lines representative of the gut and brain treated with an ethanol extract of LWDH, gene expression results did not strongly support appetite regulation as a body weight-lowering mechanism of action of the product. Further investigation into this potential mechanism is, however, highly warranted. Both gene and protein expression results using liver and muscle tissues, and cells lines representative of the liver and muscle, supported the notion that beneficial effects on fatty acid oxidation/synthesis may represent a potential body weight-lowering mechanism of action of the product, especially in muscle tissue and cells.

Overall, results from this thesis demonstrated the potential of LWDH as a natural agent against obesity and several associated complications, particularly through beneficial effects on fatty acid metabolism.

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DEDICATION

This thesis is dedicated to my immediate family. My parents, Robert and Gloria, were the main inspiration for this trek down the path of higher learning. My younger brother, Mitchell (MSc, University of Prince Edward Island; PhD Candidate, University of British Columbia), also provided me with a wealth of guidance and inspiration throughout this journey. Mitch – your future is looking undoubtedly quite bright.

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LIST OF ABBREVIATIONS

Abbreviation	Term
1°	Primary
2º	Secondary
A	Adenine
ACC	Acetyl-CoA Carboxylase
AgRP	Agouti-related Peptide
ALP	Alkaline Phosphatase
ALT	Alanine Aminotransferase
AMPK	AMP-activated Protein Kinase
AP	Ammonium Persulfate
Arc	Arcuate Nucleus
AST	Aspartate Aminotransferase
ATCC	American Type Culture Collection
BBB	Blood Brain Barrier
BCA	Bicinchoninic Acid
BID	Twice-daily
BMI	Body Mass Index
BSA	Bovine Serum Albumin
C	Cytosine
CART	Cocaine- and Amphetamine-regulated Transcript
CC	Compound C

cDNA	Complementary DNA
CNS	Central Nervous System
CPT1	Carnitine Palmitoyltransferase-1
CRP	C-reactive Protein
CVD	Cardiovascular Disease
DEPC	Diethylpyrocarbonate
dH_2O	Deionized Water
DMEM	Dulbecco's Modified Eagle Medium
ELISA	Enzyme-linked Immunosorbent Assay
EMEM	Eagle's Minimum Essential Medium
EtOH	Ethanol
FA	Fatty Acid
FAS	Fatty Acid Synthase
FBS	Fetal Bovine Serum
FC	*Fructis Corni*
FFA	Free Fatty Acid
G	Guanine
GGT	γ-Glutamyl Transferase
GHS	Growth Hormone Secretagogue Receptor
GI	Gastrointestinal
GP_x	Glutathione Peroxidase
GSH	Reduced Glutathione
GSSG	Oxidized Glutathione

H_2O_2	Hydrogen Peroxide
HDL-C	High-density Lipoprotein Cholesterol
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	Horse Radish Peroxidase
i.v.	Intravenous
IL-6	Interleukin-6
LDL-C	Low-density Lipoprotein Cholesterol
LWDH	Liuwei Dihuang
MES	2-(*N*-morpholino)ethanesulfonic acid
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NPY	Neuropeptide Y
OP	Obesity-prone
OR	Obesity-resistant
pACC	Phosphorylated ACC
pAMPK	Phosporylated AMPK
PD	Parkinson's disease
PBS	Phosphate Buffered Saline
PGC-1α	Peroxisome Proliferator-activated Receptor Gamma Coactivator-1α
POMP	Pro-opiomelanocortin
PYY	Peptide Tyrosine-Tyrosine
qPCR	Quantitative Real-time Polymerase Chain Reaction

RA	*Rhizoma Alismatis*
RD	*Rhizoma Dioscoreae*
RIPA	Radioimmunoprecipitation Assay
RNA	Ribosomal Nucleic Acid
ROS	Reactive Oxygen Species
RRP	*Radix Rehmannide Preparata*
SDS	Sodium Dodecyl Sulfate
SIRT1	Sirtuin-1
SOD	Superoxide Dismutase
SREBP-1c	Sterol Regulatory Element-binding Protein-1c
T	Thymine
T1A	High Dose (3500 mg/kg body weight) LWDH treatment
T1B	Medium Dose (1500 mg/kg body weight) LWDH treatment
T1C	Low Dose (500 mg/kg body weight) LWDH treatment
T2DM	Type 2 Diabetes Mellitus
TBARS	Thiobarbituric Acid Reactive Substances
TBST	Tris-buffered Saline with Tween
TC	Total Cholesterol
TCM	Traditional Chinese Medicine
TEMED	Tetramethylethylenediamine
TID	Thrice-daily
TG	Triglyceride
TNF-α	Tumour Necrosis Factor-α

V	Volt
WC	Waist Circumference
WAT	White Adipose Tissue
WHO	World Health Organization
wt/wt	weight/weight
Y_1	Neuropeptide Y Receptor-1
Y_5	Neuropeptide Y Receptor-5

CHAPTER 1: INTRODUCTION

1.1. Overview

The present thesis consists of a series of *in vivo* and *in vitro* experiments conducted to elucidate the anti-obesity efficacy and potential mechanism(s) of action of a traditional Chinese herbal formula and its ethanol extract.

Obesity has become an increasingly common health condition over the last several decades (James 2008) and has been referred to by many as the epidemic of the 21st century (Greenberg and Obin 2006). A greater than 70% increased risk of developing a myriad of associated complications, such as irregularities in lipid and lipoprotein metabolism, leptin and insulin resistance, abnormal liver enzyme profiles, chronic low-grade inflammation, systemic oxidative stress, and dysregulated production of various adipokines have been attributed to obesity (Rippe 1998; Haslam and James 2005). Notably, however, it has long been recognized that even modest weight loss can improve, or even abolish, the vast majority of obesity-associated complications (Goldstein 1992; Cordero-MacIntyre et al. 2000).

Currently available weight loss treatment strategies include lifestyle changes, such as diet and exercise, pharmacological intervention, and bariatric surgery (Adams et al. 2007; Klonoff and Greenway 2008; Xiao and Yang 2012). For many persons suffering from obesity, lifestyle changes often represent an ineffective and/or inadequate anti-obesity treatment strategy (Klonoff and Greenway 2008). However, well-structured interventions, such as pedometer-based physical activity (Chan et al. 2004) and the Diabetes Prevention Program (Knowler et al. 2009) have shown promising results.

Furthermore, most pharmacological interventions have been shown to be associated with limited long-term success and a variety of safety concerns (Kang and Park 2012). Despite its relative effectiveness in promoting (O'Brien et al. 2006) and maintaining (Christou and MacLean 2005) weight loss, bariatric surgery has also been rendered largely impractical as a viable anti-obesity strategy given its high cost, mortality rates, and relative invasiveness (Adams et al. 2007). Therefore, a strong demand remains to continue searching for both safe and efficacious products to combat the obesity epidemic.

Traditional Chinese medicine (TCM) has played a pivotal role in disease prevention and treatment, primarily in Asian countries (Li et al. 2008; Yin et al. 2008; Pan et al. 2010). More recently, TCM has represented a viable alternative treatment approach with enormous potential as a starting point for the development of anti-obesity products in Western countries as well (Perry et al. 2012). The ancient TCM formula, Liuwei Dihuang (LWDH), is widely produced in China and has traditionally been used for the treatment of diseases associated with "kidney-yin deficiency", such as T2DM, hyperlipidemia, and hypertension (Xie et al. 2008). Its use worldwide has been for general health promotion (Ma et al. 2004). Limited studies have demonstrated its ability to reduce visceral fat deposition in rats (Xue et al. 2006) and inhibit rat preadipocyte differentiation (Xiao et al. 2007). Overall, however, very little to no investigation into its potential benefits in regard to energy homeostasis and weight management, or their associated mechanism of action, has been documented.

To investigate the efficacy of LWDH as a potential anti-obesity product *in vivo*, a rat model of obesity representative of the human condition of obesity (Jackman et al. 2006) was employed. Chapters 2 and 3 of this thesis will outline results from this *in vivo*

study. To subsequently investigate the potential anti-obesity mechanism(s) of action of LWDH, a series of *in vivo* experiments using collected tissue samples from LWDH-treated obese rats and *in vitro* experiments using representative cell lines treated with an ethanol extract of LWDH were conducted. Chapter 4 of this thesis will outline results from *in vivo* and *in vitro* analyses investigating the effect of LWDH and its ethanol extract on appetite regulation as a potential anti-obesity mechanism of action of the product. Chapter 5 will outline results investigating the effect of LWDH and its extract on key aspects of fatty acid metabolism.

1.2. Definition of obesity

In the biological sense, obesity can be defined as an abnormal or excessive accumulation of fat in adipose tissue, to the extent that health is impaired (WHO 2000). In order to quantify weight changes across populations and estimate health and economic risks associated with obesity, simple anthropometric screening indices have included the body mass index (BMI) and measurements of waist circumference (WC) and skin fold thickness (Laurson et al. 2011). The most commonly used measurement has been the BMI (Janssen et al. 2012). The ease by which it can be calculated (weight in kilograms (kg) divided by the square of height in meters (m)), its defined risk categories (Figure 1-1; most commonly reported as overweight, BMI $\geq$ 25 kg/m^2 or obese, BMI $\geq$ 30 kg/m^2) (Prentice 2006), and its close correlation with body fat in the vast majority of people (Stein and Colditz 2004; Barlow 2007; Dietz et al. 2009) have collectively contributed to the common use of BMI for body weight quantification.

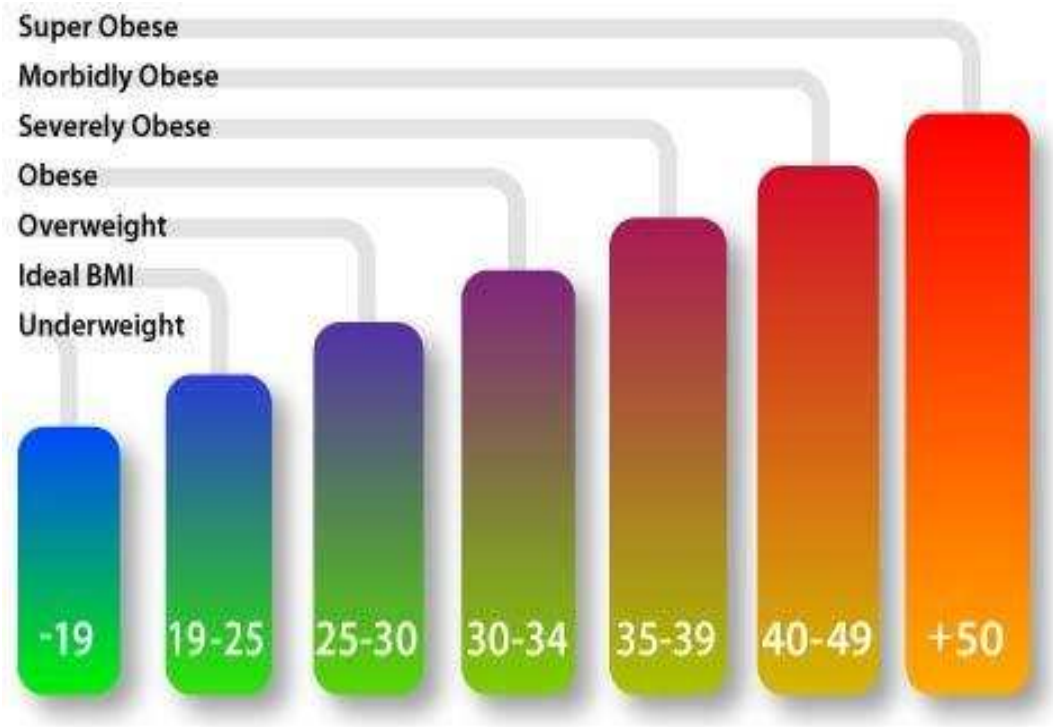


Figure 1-1: Quantification of body weight using the body mass index. Adapted from Janssen et al. (2012). "Prevalence and secular changes in abdominal obesity in Canadian adolescents and adults, 1981 to 2007-2009." Obes Rev 12(6): 397-405.

1.3. Prevalence of obesity

Obesity has become an increasingly common health condition over the last several decades and its global prevalence only continues to escalate (Drewnowski and Popkin 1997; Wang et al. 2002; James 2008). In 2009, it was estimated that this largely preventable disease affects over one billion people worldwide and that its prevalence will have at least doubled by the year 2030 (Gnacinska et al. 2009). Currently, obesity is the leading cause of preventable deaths worldwide (Alfadda and Sallam 2012) and it is predicted to become the biggest public health problem of the century (Gnacinska et al. 2009). Collectively, these reports have resulted in obesity as having been referred to as the epidemic of the 21st century (Greenberg and Obin 2006).

Closer to home, the percentage of obese Canadians has also risen dramatically (Tremblay et al. 2002), with recent estimates of an approximately 25% obese population (Janssen et al. 2012). It has been well documented, however, that the obesity epidemic is not limited to only the most affluent countries, but has been spreading to many of the less affluent countries as well (Popkin 1998; Janssen et al. 2012). Over 20% of all adults in most European countries are obese (James 2008). The Middle East now has similar rates of obesity, in addition to the highest national prevalence of T2DM in the world (James 2008). In less affluent Caribbean countries, Africa, and Asia, there has been an overwhelming rise in obesity-associated pre-mature deaths, as well as changes in diet and physical activity associated with obesity (James 2008). As the obesity epidemic continues to spread, so too have concerns about the staggering associated economic consequences. In fact, in terms of the economic burden of obesity, obese individuals have been

collectively documented to accrue upwards of 30% greater medical costs compared to their normal weight counterparts (Andreyeva et al. 2004; Lakdawalla et al. 2005).

1.4. Etiology of obesity

The interaction of several factors, including behavioural, environmental, physiological, and hereditary has contributed to the ever increasing prevalence of obesity in recent years (Stein and Colditz 2004). Notably, the alarming rate at which the prevalence statistics have increased has been reported to be suggestive of a more significant contribution of a combination of behavioural and environmental factors toward the obesity epidemic (Stein and Colditz 2004). Collectively, increased energy intake and decreased energy expenditure have given rise to the marked increases in body weight in our society over the last several decades (Harnack et al. 2000). At least in part responsible for this shift towards a positive energy balance have been increases in fast food and soft drink consumption (Harnack et al. 2000; French et al. 2003), increasingly larger portion sizes (Smiciklas-Wright et al. 2003), and decreases in physical and increases in sedentary activities (Jeffery and Utter 2003; Haslam and James 2005).

1.5. Obesity-associated risks/complications

Obesity has been reported to negatively affect physical functioning and the general quality of human life (Coakley et al. 1998; Fine et al. 1999), and has also been associated with a reduction in average life expectancy (Hu et al. 2001). Furthermore, even small increases in body weight have been documented to confer a significant negative impact on overall public health (Stein and Colditz 2004). Excess body weight

increases the risk of developing a myriad of serious chronic diseases, including, but not limited to, type 2 diabetes mellitus (T2DM), cardiovascular disease (CVD), and certain forms of cancer (Mokdad et al. 2003). Furthermore, it has been reported that a BMI score of greater than 27 kg/m^2 predisposes to a 70% greater risk of developing a suite of obesity-associated complications (Rippe 1998). Importantly, this risk can manifest not only in overweight and obese individuals, but also in those at the upper end of the normal body weight (20 $kg/m^2 \geq$ BMI $\geq$ 25 kg/m^2) range (Field et al. 2001). Noteworthy, however, is the evidence that even modest losses in body weight have long been documented to improve, or even reverse, the vast majority of obesity-associated complications (Goldstein 1992; Cordero-MacIntyre et al. 2000).

For the purpose of this thesis, the relationship between excess body weight and lipid and lipoprotein irregularities, leptin and insulin resistance, liver enzyme profile abnormalities, chronic low-grade inflammation, systemic oxidative stress, and dysregulated adipokine production will be discussed. Subsequent chapters of this thesis will provide evidence of the associated beneficial effects of body weight control by dietary supplementation of LWDH on these associated complications.

1.5.1. Lipid and lipoprotein irregularities

Excess body weight has long been known to result in changes in circulating levels of lipids and lipoproteins (Larsson et al. 1984; Flock et al. 2011). Obesity has been reported to exacerbate various dyslipidemias, such as elevated total cholesterol (TC), triglyceride (TG), and low-density lipoprotein cholesterol (LDL-C) levels, as well as diminished high-density lipoprotein cholesterol (HDL-C) levels (Ferrannini et al. 1991;

Cordero-MacIntyre et al. 2000) (Figure 1-2). Furthermore, being overweight or obese, in addition to having a lipid irregularity, confers a greater risk of developing CVD (Sharma 2003), which remains to be the leading cause of mortality in developed countries (Kung et al. 2008). Conversely, weight loss has been shown to improve irregularities in lipid and lipoprotein concentrations (Larsson 1991; Dattilo and Kris-Etherton 1992). Levels of TC, TG, and LDL-C have been shown to be reduced in response to weight loss (Despres et al. 1989; Peeples et al. 1989; Weinsier et al. 1992), while HDL-C levels have been shown to be increased in response to the same effect (Dattilo and Kris-Etherton 1992). More recently, non-HDL-C, commonly calculated as TC minus HDL-C and accounting for LDL-C and other lipoprotein fractions, has been documented to better represent CVD risk than LDL-C alone, despite being recognized only as a secondary therapeutic target in persons with elevated TG levels (Hoenig 2008). Furthermore, non-HDL-C has even been documented as a superior CVD risk predictor than LDL-C alone, regardless of TG levels (Liu et al. 2006). Increased BMI scores have been shown to be associated with elevated non-HDL-C levels, which are also known to correlate inversely with HDL-C levels (Srinivasan et al. 2002). Notably, however, weight loss has been shown to be associated with improved non-HDL-C levels in obese individuals (Wong et al. 2011).

In addition to the above mentioned obesity-associated irregularities in blood lipids and lipoprotein fractions, circulating free fatty acid (FFA) levels are typically elevated in the obese state, in response to a combination of enlarged adipose tissue mass, increased FFA release, and/or reduced FFA clearance (Boden 2008). Elevated FFA levels in obesity are well documented to be associated with insulin resistance (Boden 1997; Kahn et al. 2006), making them a likely culprit in the development of T2DM (Wilding 2007).

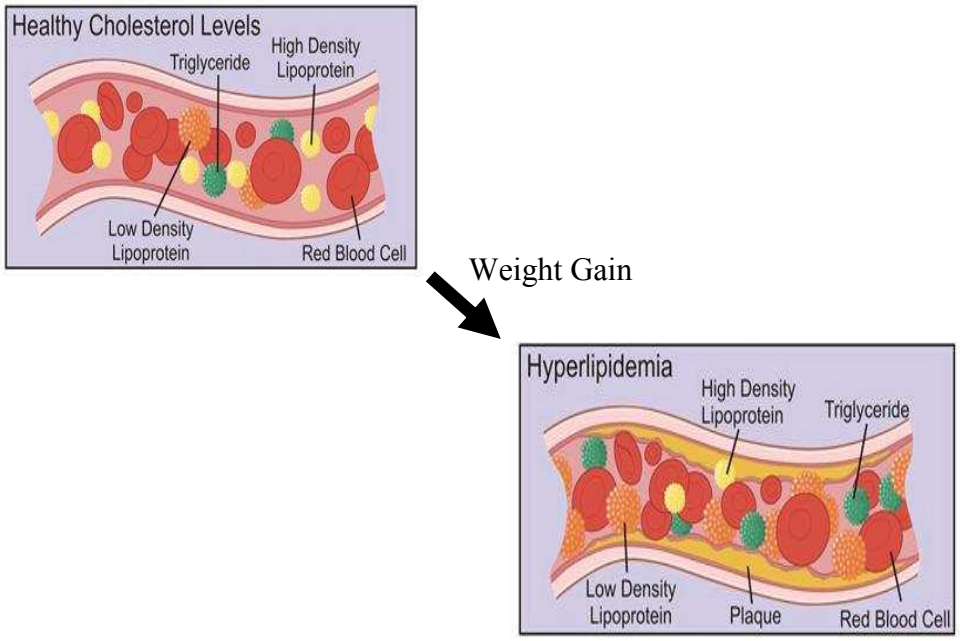


Figure 1-2: Relationship between weight gain and lipid and lipoprotein irregularities. Adapted from Cordero-MacIntyre (2000). "Weight loss is correlated with an improved lipoprotein profile in obese postmenopausal women". JACN 19(2): 275-284.

Weight loss, however, has been shown to be associated with a reduction in FFA levels and an improvement in insulin sensitivity (Goodpaster et al. 1999; Boden 2008).

1.5.2. Leptin and insulin resistance

Leptin, secreted exclusively by white adipose tissue (WAT), represents one of the core components of the physiological control of body weight (Murphy and Bloom 2004). Leptin inhibits food intake, increases energy expenditure, and reduces lipid accumulation in tissues by stimulating fatty acid oxidation and inhibiting lipogenesis (Unger 2003). Leptin levels have been shown to be positively correlated with body fat mass (Kelesidis and Mantzoros 2006). However, in most cases of obesity, despite high circulating levels and intact receptors, there is a diminished response to the beneficial effect of leptin, suggestive of the development of leptin resistance in the obese state (Enriori et al. 2007). Notably, exercise-driven weight loss has been shown to largely prevent the obesity-induced development of leptin resistance (Steinberg et al. 2004). Furthermore, both diet- and exercise-driven weight loss alike have been shown to improve leptin sensitivity (Steinberg and Dyck 2000). In addition, moderate weight loss of as little as 5% has been reported to lower leptin to near physiological levels, resulting in improved sensitivity to its beneficial effects (Mazzali et al. 2006)

Insulin resistance, a state in which a given amount of insulin produces a below average physiological response (Kahn 1978; Kahn et al. 2006), has been reported to strongly correlate with increased adiposity (Caprio 2002), and in particular, increased circulating levels of FFA (Kahn et al. 2006). More specifically, insulin resistance refers to the reduced ability of insulin to stimulate glucose use in the peripheral tissues and to

suppress glucose production in the liver (Matthaei et al. 2000). Indeed, insulin resistance has been documented as the most common obesity-associated metabolic complication (Weiss and Kaufman 2008). Furthermore, insulin resistance is considered to represent an important link between obesity and several associated metabolic complications, particularly T2DM (Alberti et al. 2004). Weight loss has been shown to improve insulin sensitivity in both healthy and diabetic individuals (Goodpaster et al. 1999). Furthermore, a significantly reduced risk of developing insulin resistance and T2DM has been shown in response to losses of body weight of as little as 5-7% (Tuomilehto et al. 2001).

1.5.3. Liver enzyme profile abnormalities

Abnormal liver enzyme levels have been reported to be indicative of liver damage or alterations in bile flow (Giannini et al. 2005). Several common biomarkers of such pathologies include aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), and γ-glutamyl transferase (GGT). Studies have shown that increased circulating levels of these biomarkers are often associated with obesity (Dixon et al. 2001; Iacobellis et al. 2007) and increase the risk of developing obesity-associated complications, such as CVD and T2DM (Hanley et al. 2004; Stefan et al. 2008). A mild increase in aminotransferase (AST or ALT) enzyme levels and/or an AST/ALT ratio greater than one are the most commonly documented liver enzyme alterations (Giannini et al. 2005) and have been associated with excess body fat mass and obesity (Marchesini et al. 2001). An increase in ALP is typically indicative of cholestasis, or bile duct obstruction (Moss 1997), and has also been shown to be independently associated with obesity (Nannipieri et al. 2005). Similar to ALP, GGT is also commonly elevated in

response to bile duct damage or obstruction (Giannini et al. 2001), and increased levels have been linked to obesity, physical inactivity, dyslipidemia, and other obesity-associated co-morbidities (Wannamethee et al. 1995). Due to its relative lack of specificity yet high sensitivity for liver damage, GGT is also often useful for further elucidation of altered ALP levels, or in combination with AST/ALT measurements, for more specifically diagnosing liver damage (Giannini et al. 2005). Lifestyle modifications, such as increased physical activity and decreased alcohol consumption, with corresponding weight loss have been shown to reduce circulating liver aminotransferase levels in obesity (Suzuki et al. 2005; Utzschneider and Kahn 2006). Similarly, weight reduction as a result of low-fat diet feeding in obese individuals has been shown to significantly lower serum levels of AST, ALT, and GGT (de Luis et al. 2010). A reduction in serum liver enzymes levels, most notably ALT, has also been largely attributed to weight loss in obese diabetic subjects (Krakoff et al. 2010).

1.5.4. Chronic low-grade inflammation

Adipose tissue is no longer considered to be a passive storage depot for excess fat (Flier 1995), but rather, is now known to produce several inflammatory mediators (Browning et al. 2008). Indeed, obesity has been shown to be associated with a state of chronic low-grade inflammation (O'Rourke 2009) (Figure 1-3). Elevated levels of circulating inflammatory biomarkers common to obesity include the pro-inflammatory cytokine tumour necrosis factor α (TNF-α), the acute phase pro-inflammatory cytokine interleukin-6 (IL-6), and the chronic inflammatory biomarker C-reactive protein (CRP)

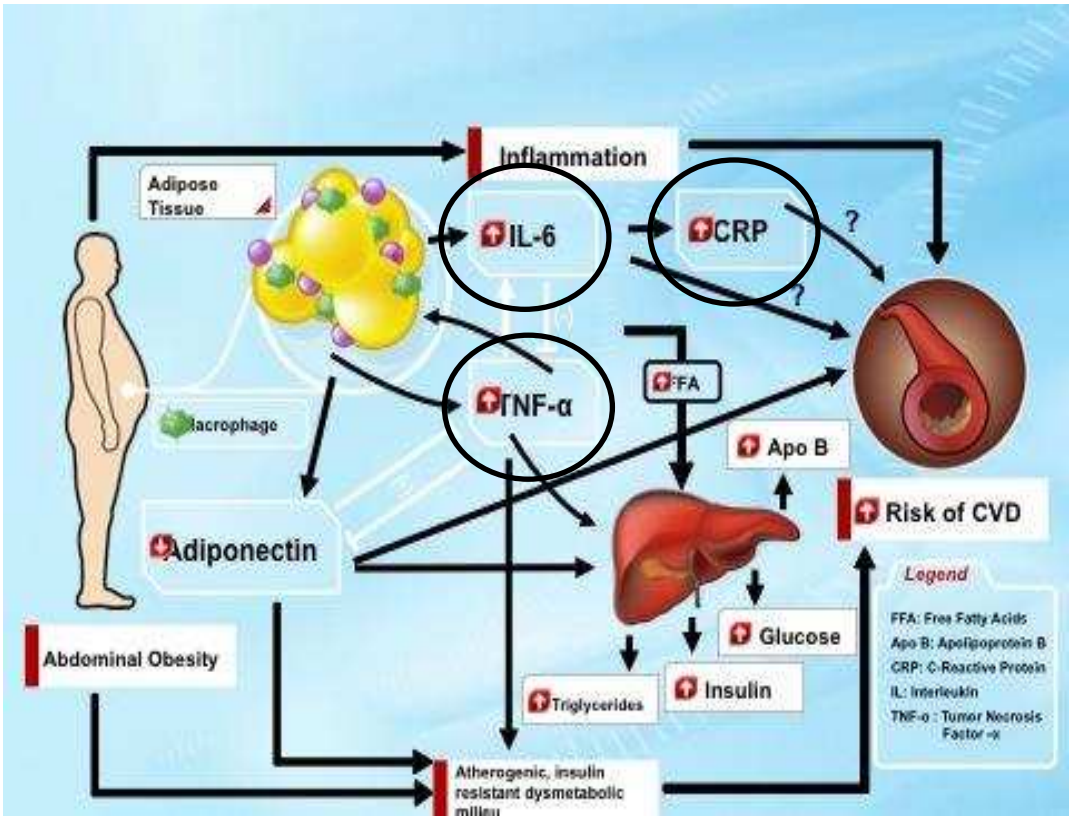


Figure 1-3: Association between obesity and circulating biomarkers of inflammation. Adapted from Despres (2003). "Insulin resistance and abdominal adiposity in young men with documented malnutrition during the first year of life". Int J Obes Metab Disord 27: 5224.

(Visser et al. 1999; Kern et al. 2001). In addition to being increased in the obese state, inflammation has been investigated as a possible contributor to several of the most serious obesity-related co-morbidities (O'Rourke 2009). It is evidenced that both diet- and surgery-induced weight loss have been shown to be effective in reducing obesity-related inflammation (Laimer et al. 2002; Ziccardi et al. 2002) and improving several obesity-associated co-morbidities (Krebs et al. 2002).

The pro-inflammatory cytokine TNF-α is constitutively expressed in adipocytes (Hotamisligil et al. 1995). Its expression has been shown to be upregulated in response to obesity (Hotamisligil et al. 1995) and in proportion to the degree of adiposity (Bullo et al. 2002). Furthermore, levels of TNF-α in the circulation have been shown to be increased in obese compared to lean individuals (Dandona et al. 1998). Adipose tissue expression of TNF-α has, however, been shown to decrease in proportion to body weight loss (Hotamisligil et al. 1995; Bullo et al. 2002). Circulating levels of TNF-α have also been shown to decrease following weight loss and/or an increase in physical activity (Kern et al. 1995; Dandona et al. 1998).

Compared to lean individuals, adipose tissue of obese persons has been shown to elicit an increase in the expression of the acute phase pro-inflammatory cytokine IL-6, which has been shown to occur in proportion to body fat mass (Vozarova et al. 2001). In fact, adipose tissue IL-6 expression has been reported to be 10-fold higher in obese compared to lean individuals (Fried et al. 1998). Similarly, levels of IL-6 in the circulation have also been shown to increase with obesity and as much as 30% of which has been reported to be derived from adipose tissue (Mohamed-Ali et al. 1997). A

reduction in body fat mass has, however, been shown to result in both reduced adipose tissue expression and circulating levels of IL-6 (Kershaw and Flier 2004).

Reportedly the most extensively studied inflammatory biomarker, CRP is produced mainly by the liver, largely in response to obesity-induced inflammation (Nguyen et al. 2009). Given its stable half-life and lack of diurnal variation in the blood, CRP has been reported as the best characterized biomarker of chronic inflammation (Devaraj et al. 2010; Punyadeera et al. 2011). Changes in CRP have been shown to significantly correlate with changes in adiposity (Browning et al. 2008). Indeed, obese individuals have increased circulating levels of CRP as compared to their lean counterparts (Kopp et al. 2003). Furthermore, CRP is a strong predictor of obesity-associated co-morbidities; even stronger than IL-6, the cytokine which stimulates its production (Pradhan et al. 2001). Significant reductions in CRP have, however, been shown in response to moderate weight loss and bariatric surgery (Vazquez et al. 2005; Selvin et al. 2007).

1.5.5. Systemic oxidative stress

It has been shown that reactive oxygen species (ROS) are positively correlated with BMI (Keaney et al. 2003) and elevated in the obese state, resulting in increased oxidative stress in accumulated fat (Furukawa et al. 2004) (Figure 1-4). Furthermore, an increase in ROS from accumulated fat has been shown to lead to increased systemic oxidative stress and play a critical role in the onset of several obesity-associated co-morbidities (Melissas et al. 2006). In the obese state, the main source of ROS production is believed to be NADPH oxidase, which catalyzes the reduction of oxygen to yield

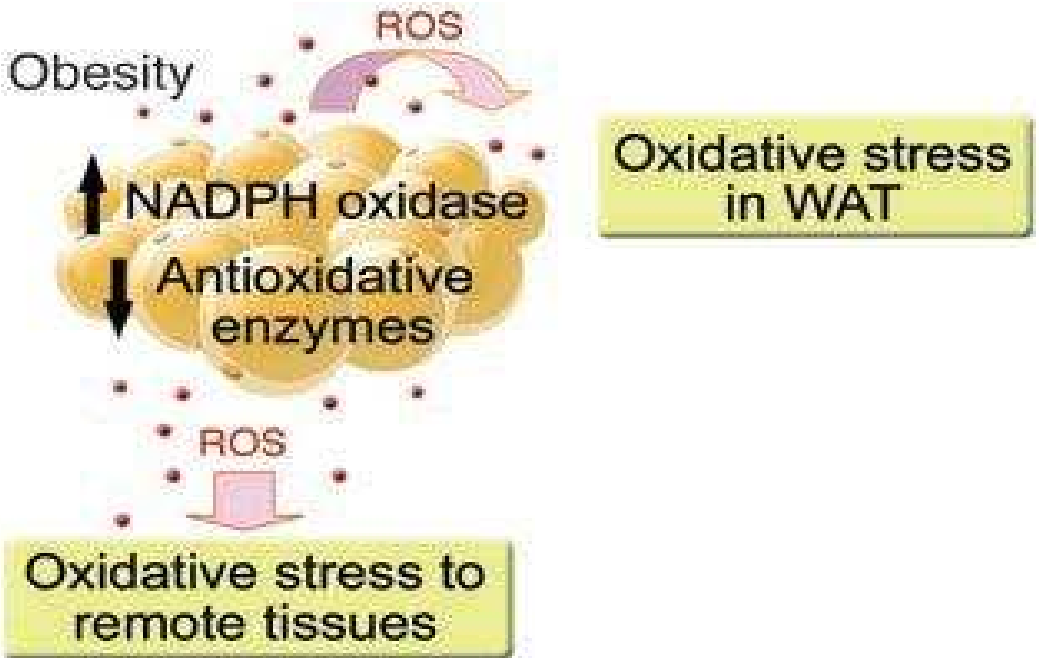


Figure 1-4: Association between obesity and systemic oxidative stress. Adapted from Furukawa (2004). "Increased oxidative stress in obesity and its impact on metabolic syndrome." J Clin Invest 114(12): 1752-61.

superoxide anions, with NADPH serving as the electron donor (Feillet-Coudray et al. 2009). When produced at chronically high levels, ROS can have detrimental effects through irreversible oxidation of their primary targets, including lipids, proteins, and DNA (Valko et al. 2007). In addition to increased ROS production and resultant systemic oxidative stress, obesity has also been shown to be associated with a decline in antioxidant defences (Khan et al. 2006), including suppressed activity of the antioxidant enzyme superoxide dismutase (SOD) and lower levels of glutathione (GSH) (Furukawa et al. 2004). Furthermore, adipose tissue of obese subjects has been shown to produce increased levels of thiobarbituric acid reactive substances (TBARS), a marker of lipid peroxidation and oxidative damage (Furukawa et al. 2004). Caloric restriction and reduced adiposity have, however, been shown to be associated with improvements in circulating biomarkers of oxidative stress and antioxidant defences (Heilbronn et al. 2006).

In order to protect against the potentially detrimental effects of ROS, cells are equipped with antioxidant defence systems (Feillet-Coudray et al. 2009). Superoxide dismutase is considered the first line of defence against oxidative stress (Johnson 2002) and plays a physiological antioxidative stress role by converting superoxide anions to hydrogen peroxide (H_2O_2), which can then be further detoxified through the action of glutathione peroxidase (GP_x) (Fridlyand and Philipson 2005). Adiposity and increased caloric intake alike have been shown to result in decreased activity of SOD (Beltowski et al. 2000). Inversely, weight loss and exercise training have been shown to result in increased SOD activity and a resultant improvement in the first line of antioxidant defence (Selamoglu et al. 2000; Shih et al. 2006).

Glutathione is a key antioxidant that plays a major role in maintaining redox balance by providing reduced ROS for GP_x-catalyzed H_2O_2 reductions (Feillet-Coudray et al. 2009). Furthermore, the ratio between reduced (GSH) and oxidized (GSSG) glutathione is a strong and reliable biomarker of oxidative stress (Ashfaq et al. 2006). Levels of GSH have been shown to be significantly reduced in response to obesity (Feillet-Coudray et al. 2009). However, weight loss has been shown to largely reverse this effect (Uzun et al. 2007).

Lipids are among the main classes of molecules targeted by ROS (Joao Cabrera et al. 2010) and lipid peroxidation has been shown to be associated with increased adiposity (Noeman et al. 2011). Obesity-associated lipid peroxidation is believed to be caused by at least one of three mechanisms, including increased ROS production, cell injury as a result of increased body mass pressure, and/or consumption of a high fat diet (Olusi 2002). The major indicator of lipid peroxidation is malondialdehyde (MDA), levels of which can be determined by measuring thiobarbituric acid reactive substances (TBARS) (Uzun et al. 2007). Obesity is associated with an increase in lipid peroxidation, evidenced in large part by increases in MDA and TBARS levels (Furukawa et al. 2004; Noeman et al. 2011). Fortunately, weight loss of as little as 5-10% through either caloric restriction or surgical intervention has been shown to lower MDA and TBARS levels in obese patients (Dandona et al. 2001; Mohn et al. 2005).

1.5.6. Dysregulated adiponectin production

Adipose tissue is now regarded as an active endocrine organ which produces and secretes a variety of bioactive mediators termed adipokines (Rabe et al. 2008).

Adiponectin represents the most abundant circulating adipokine and is secreted exclusively from adipose tissue (Schraw et al. 2008). It is a 30kD molecule existing in at least 3 different complexes, including a high molecular weight (HMW) form (12-36 mer), a low molecular weight (LMW) form (hexamer), and a trimeric form (trimer) (Pajvani et al. 2003). Adiponectin exerts its effects via its specific receptors, AdipoR1 and AdipoR2, on insulin-sensitive cells in liver, muscle, and adipose tissues (Sattar and Sattar 2012). Circulating levels of adiponectin have been established as an important biomarker for several metabolic disorders (Lara-Castro et al. 2006), in addition to playing a role in the control of lipid and glucose metabolism (Berg et al. 2001). HMW adiponectin in particular has been linked to increased fat oxidation in muscle tissue and improved whole-body insulin sensitivity (Fruebis et al. 2001; Yamauchi et al. 2001). Unlike the vast majority of adipokines, circulating adiponectin levels are decreased in response to obesity (Kadowaki and Yamauchi 2005). A negative correlation between circulating adiponectin levels and obesity has been shown in both humans and rodent models (Arita et al. 1999; Hotta et al. 2001), with particularly strong correlations in response to visceral obesity (Cnop et al. 2003; Yatagai et al. 2003). Increases in circulating adiponectin levels have been shown in response to weight loss (Martos-Moreno et al. 2010) and bariatric surgery (Illan-Gomez et al. 2012). Exercise training has also been shown to beneficially affect adiponectin levels, independent of weight loss or gain (Lee et al. 2012).

1.6. Obesity treatment/management

Given the interrelated combination of behavioural, environmental, physiological, and hereditary factors that contribute to obesity, prevention, treatment, and/or management of this complex disease has proven extremely difficult (Kushner and

Weinsier 2000). Currently available weight loss treatment strategies include lifestyle changes, such as dietary and exercise intervention, pharmacological intervention, and bariatric surgery (Xiao and Yang 2012). It is generally accepted that primary treatment of obesity should include lifestyle changes, with a focus on behavioural and environmental modification (Stafford and Radley 2003). Dietary and exercise changes have in large part focused on reducing calorie intake, such as by adhering to a low-fat diet (Samaha et al. 2003; Vetter et al. 2010) and increasing the amount of daily physical activity (Jakicic et al. 1999; Jeffery and Utter 2003). Resistance training has also been shown to be beneficial for maintaining fat-free mass during weight loss (Jakicic et al. 1999). In obese patients presenting with associated co-morbidities, or in cases where there is minimal or no response to primary treatment, pharmacological intervention is often considered as a secondary treatment option (Greenberg et al. 1999; Snow et al. 2005). Lastly, in morbid obesity (BMI $\geq$ 40 kg/m^2), or in obesity (35.0 kg/m^2 $\geq$ BMI $\geq$ 39.9 kg/m^2) accompanied by an associated co-morbidity, bariatric surgery has been shown to be a highly effective and relatively safe treatment strategy (Sjostrom et al. 2012).

1.6.1. Primary treatment: lifestyle changes

Weight loss is essential for the prevention, treatment, and management of obesity (Renehan et al. 2008). Accordingly, it has been reported that diet- and exercise-driven primary anti-obesity intervention should be targeted at an approximate 10% loss in initial body weight (WHO 2000). For the majority of obese persons, short-term weight loss is achieved more readily through dietary intervention than through increased physical activity (Xiao and Yang 2012). In fact, many obese persons can achieve short-term weight loss through dietary intervention alone (Xiao and Yang 2012). Although

achieving long-term weight loss success is more difficult, managing food intake remains to be the focal point of first-line anti-obesity treatment (Xiao and Yang 2012). Physical activity is associated with a marked increase in energy expenditure (Bruce et al. 2006). Although exercise alone is generally not a sufficiently effective short-term weight loss strategy, when combined with dietary intervention, it represents an integral component of long-term weight loss success (Xiao and Yang 2012).

Upwards of twenty percent of overweight or obese persons show long-term weight loss success (Wing and Phelan 2005). Well-designed lifestyle interventions have been particularly effective. In Prince Edward Island, a pedometer-based physical activity intervention resulted in increased physical activity in sedentary workers (Chan et al. 2004). Other major randomized trials, such as the Diabetes Prevention Program (Knowler et al. 2002; Knowler et al. 2009), the Finnish Diabetes Prevention study (Tuomilehto et al. 2001), and the look AHEAD (Action for Health in Diabetes) (Pi-Sunyer et al. 2007) have shown promising results in response to intensive lifestyle intervention. In comparing results from lifestyle versus pharmacological intervention, 10-year follow-up results from the Diabetes Prevention Program Outcomes Study (Knowler et al. 2009) demonstrate that lifestyle intervention can be superior to pharmacological intervention in promoting and maintaining weight loss. However, in this particular study, modest weight loss in the long-term was achieved more readily by way of pharmacological intervention. Collectively, weight loss and weight maintenance by way of lifestyle changes are associated with a reduced risk for the development of the most serious obesity-associated co-morbidities and a general improvement in the overall quality of life (Thompson et al. 1999; Caterson et al. 2012).

1.6.2. Secondary treatment: pharmacological intervention

For many persons suffering from obesity, lifestyle changes such as dietary habits and exercise often represent an ineffective and/or inadequate anti-obesity treatment strategy (Klonoff and Greenway 2008). For obese persons, or overweight persons also suffering from an obesity-associated complication, pharmacological intervention as a complement to lifestyle changes is often times a viable option for the induction, and furthermore, the maintenance of weight loss (Snow et al. 2005). Unfortunately, most pharmacological interventions are associated with poor adherence rates and limited long-term success (Hemo et al. 2011), safety concerns (Ling et al. 2013), and in some cases, weight regain upon cessation of treatment (Kang and Park 2012).

1.6.2.1. Current anti-obesity drug marketplace

Anti-obesity drugs are generally classified into one of three groups, depending on their mechanism of action, and include appetite suppressors, dietary fat absorption inhibitors, and energy expenditure stimulators (Li and Cheung 2009). Various drugs and drug combinations have been used to treat and/or manage obesity over the last couple of decades, however, most of them have been removed from the market due to reports of a variety of serious adverse side effects (Li and Cheung 2011). The three most widely prescribed anti-obesity drugs over the last couple of decades include phentermine, sibutramine, and orlistat (Klonoff and Greenway 2008). The following sections will outline the rise and fall over the last couple of decades of the most common anti-obesity therapeutics.

The appetite suppressor phentermine has been in clinical use for over 50 years (Bays et al. 2007). In the past, phentermine has been prescribed as an anti-obesity

treatment in combination with other medications, most notably fenfluramine (Vetter et al. 2010). However, due to associations with cardiac valvulopathy, fenfluramine was removed from the market in 1997 (Connolly et al. 1997). Phentermine, however, was not implicated in the cardiac risks associated with the drug combination and remains to be the most commonly prescribed anti-obesity pharmacological option for short-term use (Bays et al. 2007). Chemically related to amphetamine, phentermine is approved only for short-term (up to twelve weeks) use, due in large part to its reported potential of abuse (Silverstone 1992). Other reported side effects include headache, insomnia, and mild increases of blood pressure and heart rate (Klonoff and Greenway 2008).

The noradrenaline and serotonin re-uptake inhibitor, sibutramine, was approved as a long-term anti-obesity treatment over a decade ago (Halford et al. 2007). This drug functions primarily by suppressing appetite and inducing satiety (Nisoli and Carruba 2000). Reported side effects include headache, nausea, and increased blood pressure (Klonoff and Greenway 2008). More recently, long-term use of sibutramine has been linked to severe cardiovascular risk, resulting in its withdrawal from the market in late 2010 (Li and Cheung 2011).

The gastrointestinal lipase inhibitor, orlistat, prevents lipolysis of TG into absorbable FFA, resulting in increased fecal excretion of undigested fat (Drew et al. 2007) and a corresponding reduction in the absorption of dietary fat by up to 30% (Hauptman 2000). Due to its low bioavailability (< 1%), side effects of orlistat are less reported compared to other anti-obesity drugs and usually occur in the gastrointestinal tract (Drew et al. 2007). Some concerns have been reported that orlistat may lower absorption of fat soluble dietary vitamins (McDuffie et al. 2002). As a result, adequate

vitamin supplementation is a common requirement associated with the use of orlistat (Li and Cheung 2011).

Despite that three anti-obesity drugs have been approved and used short/long-term for the last 20 years, at present only orlistat is an approved pharmacological option for long-term use. In the summer of 2012, two new anti-obesity drugs were approved, including an appetite suppressing monotherapeutic and a drug combination.

The monothereapeutic appetite suppressing agent, lorcaserin (Belviq®), has been shown to induce modest, yet significant, weight loss compared to placebo control and appears to be additionally beneficial in regard to weight loss maintenance (Thomsen et al. 2008). A short-term study on lorcaserin in both men and women reported progressive weight losses over placebo control within as little as two weeks, even despite the lack of a concurrent caloric restriction regime or increase in physical activity (Smith et al. 2009). Furthermore, short-term lorcaserin-associated weight loss has been linked to decreases in several biomarkers of cardiovascular risk (Smith et al. 2009). Longer-term studies have reported significant reductions in body weight in lorcaserin-treated obese persons compared to placebo control, attributed in large part to decreased energy intake (Martin et al. 2011). The most commonly reported side effects have been minor, including headache, nausea, and dizziness (Smith et al. 2009; Smith et al. 2010). Unlike fenflurmine, which has a similar mechanism of action but lacks the selectivity of lorcaserin, deleterious drug-related effects on cardiac valvulophathy have not been anticipated (Jones 2009). In fact, lorcaserin treatment in obese persons for up to two years

has been reported without documented risk of harmful effects on heart valve function (Smith et al. 2010).

As a complement to lifestyle changes, Qsymia®, a combination of phentermine and the anticonvulsant agent topiramate, has also been found to induce significant weight loss and to promote the maintenance of weight loss (Vetter et al. 2010; Mercer 2011). Clinical trials have reported upwards of 9% placebo-adjusted weight losses over a one year period, of which 60% of participants lost greater than 10% of their initial body weight (Kennett and Clifton 2010). The precise mechanism of action of Qsymia® is currently under investigation, but has been anticipated to reflect the mechanisms of the individual components (Mercer 2011), primarily including phentermine-induced appetite suppression (Rothman and Baumann 2009). Although the body weight-lowering mechanism of action of topiramate is still unclear, it is known to have a satiating effect (Mercer 2011). Adverse side effects of Qsymia® reported during clinical trials include headache, dizziness, dry mouth, and constipation (Gadde and Allison 2009; Vetter et al. 2010). Other safety concerns, including rare psychiatric events, have been linked almost exclusively to the topiramate component of the combinatorial treatment (Kennett and Clifton 2010).

1.6.3. Bariatric surgery

Most current weight loss approaches, including lifestyle changes and pharmacological intervention, have proven largely unsuccessful in cases of morbid (BMI $\geq$ 40 kg/m^2) obesity (Ramsey-Stewart 1995). As a result, this has spurred investigation into and the development of several bariatric surgical techniques (Colquitt et al. 2009). In

the United States alone, there has been a 10-fold increase in the number of bariatric surgeries in the past decade (Tice et al. 2008). The surgery is generally done by either gastric restriction or biliopancreatic bypass, resulting in nutritional maldigestion or malabsoption, respectively (Marceau et al. 1998; Wolfe and Morton 2005). Evidence suggests that successful short- and long-term weight loss in morbidly obese subjects can be readily achieved by bariatric surgery (O'Brien et al. 2006; Colquitt et al. 2009). Furthermore, bariatric surgery can be more effective than dieting in producing sustained weight loss (Christou and MacLean 2005). In addition to morbid obesity, the surgery is often considered in obese (35.0 $kg/m^2 \geq$ BMI $\geq$ 39.9 kg/m^2) people suffering from a concurrent obesity-associated co-morbidity that may be improved through weight loss (Brolin 2002). Although there is evidence of substantial savings in prescription medication costs following bariatric surgery-assisted weight loss (Craig and Tseng 2002), access to the procedure continues to represent a major limitation. Recent statistics showed a mean investment of between $17,000 and $26,000 per operation (Cremieux et al. 2008). In addition, early and late mortality rates associated with bariatric surgery, although low, act as a deterrent for a sub-set of eligible candidates (Buchwald et al. 2007). Overall, bariatric surgery has been shown to significantly improve obesity and several associated co-morbidities, yet the substantial cost, surgery-associated mortality rates, and relative invasiveness associated with the procedure itself have rendered the approach largely impractical as a viable anti-obesity strategy (Adams et al. 2007).

1.7. Traditional Chinese medicine (TCM)

Although lifestyle changes continue to represent the primary intervention against obesity and its associated co-morbidities, mediocre long-term compliance has greatly challenged its effectiveness (Klonoff and Greenway 2008; Li and Cheung 2011). Furthermore, very few pharmacological interventions have been successful in the prevention or treatment of obesity and bariatric surgery is generally reserved for a subset of the morbidly obese population (Adams et al. 2007; Colquitt et al. 2009). Therefore, continued investigation into the development of both safe and efficacious anti-obesity products continues to be a top priority in nutritional and medical research.

Traditional Chinese medicine (TCM) has played a pivotal role in disease prevention and treatment in Asian countries, particularly China (Li et al. 2008), and now represents a viable complementary and alternative treatment approach. It is also considered to have enormous potential as an information source and starting point for the development of anti-obesity products (Perry et al. 2012). As such, a plethora of traditional Chinese herbs and their bioactive components have been, and will continue to be, investigated for their beneficial anti-obesity effects (Yin et al. 2008). Common examples of such Chinese herbal medicines include ginseng, which has been shown to have beneficial effects on glucose and lipid metabolism, as well as food intake regulation (Kim and Park 2003; Kim et al. 2005; Vuksan et al. 2008), and the plant alkaloid berberine, which has also been intensely investigated for its beneficial effects on glucose and lipid metabolism (Kong et al. 2004; Wang et al. 2011).

1.7.1. Liuwei Dihuang (LWDH)

One of the more common approaches to TCM is herbology, a Chinese art of combining various medicinal herbs into a single therapeutic prescription (Yin et al. 2008). Recently, a number of studies have been conducted to investigate the efficacy and mechanism of action of several medicinal herbs and herbal formulations on body weight and composition (Lenon et al. 2012; Sui et al. 2012). The TCM of interest for this thesis was the ancient herbal formula Liuwei Dihuang (LWDH). LWDH is prepared from a combination of six Chinese herbs (Table 1-1), including *Radix Rehmanniae Preparata*, *Rhizoma Dioscoreae*, *Fructis Corni*, *Cortex Moutan*, *Rhizoma Alismatis*, and *Poria* (Xie et al. 2008). It is widely produced in China and commonly used for the treatment of diseases associated with "kidney-yin deficiency" (Ma et al. 2004). LWDH has been used worldwide for general health promotion (Ye et al. 2009) and its safety has been evaluated (Ha et al. 2011). Various studies have demonstrated its anti-aging, anti-T2DM, and radical-scavenging effects (Hsieh et al. 2003; Ye et al. 2009). Despite reports of its effect on reducing visceral fat deposition in rats and inhibiting rat preadipocyte differentiation (Xue et al. 2006; Xiao et al. 2007), few investigations into its potential anti-obesity effects have been documented.

Table 1-1: Composition of Liuwei Dihuang herbal formula.

Herb	Source	Amount (g)
Radix Rehmanniae Praeparata	Root of *R. glutiosa*	160
Fructis Corni	Fruit of *C. officianalis*	60
Cortex Moutan	Root bark of *P. suffruticosa*	60
Rhizoma Dioscoreae	Rhizome of *D. opposita*	80
Poria	Sclerotia of *P. cocos*	60
Rhizoma Alismatis	Rhizome of *A. plantago-aquatica*	60

Adapted from Xie et al. (2008). "An approach based on HPLC-fingerprint and chemometrics to quality consistency evaluation of Liuwei Dihuang pills produced by different manufacturers". J Pharm Biomed Anal 48(4): 1261-1266.

1.7.2. Liuwei Dihuang (LWDH) ethanol extract

As LWDH is a crude mixture of medicinal herbs, information on the bioactive components potentially responsible for its body weight-lowering effect is lacking. Furthermore, the potential mechanism of action of LWDH on obesity remains to be elucidated. In order to further investigate the potential body weight-lowering efficacy and mechanism of action of LWDH, an ethanol extract of the product was investigated using *in vitro* experimental designs. Unpublished data from the Wang lab has indicated that *in vivo*, the ethanol extract of LWDH reduces weight gain and visceral fat accumulation, as well as improves metabolic phenotypes such as lipid profiles and leptin levels. This same study also revealed beneficial effects of the ethanol extract on fat and carbohydrate oxidation, as well as energy expenditure.

1.8. Appetite regulation and body weight control

The body has evolved mechanisms to control body weight over time, however, it tends to favour defending against starvation and are intuitively less robust in preventing obesity (Chaudhri et al. 2008). Furthermore, the sedentary lifestyle that has in part fuelled the now well recognized obesity epidemic, has turned this evolutionary advantage of storing excess energy as fat into an increased risk of morbidity or mortality (Roth and Reinehr 2010). Thus, harnessing of the physiological mechanism of appetite regulation has recently become an intensely investigated approach to body weight control and has been reported to involve a communication line from the gastrointestinal (GI) tract to the central nervous system (CNS) and resident centres of appetite regulation (Gardiner et al. 2008).

1.8.1. The gut/brain axis

Increasing recognition of the intricate interplay between gut hormones and the CNS has resulted in enhanced understanding of the control of food intake and body weight through appetite regulation (Hameed et al. 2009). As such, this line of communication between the GI tract and the CNS, referred to as the gut-brain axis, has been recognized as a key component of a now well-established model of appetite regulation (Chaudhri et al. 2008). The gut-brain axis has both hormonal and neural components which relay information to key CNS centres, particularly the hypothalamus (Stanley et al. 2005) (Figure 1-5). The arcuate nucleus (Arc) represents the primary CNS region involved in the control of food intake (Valassi et al. 2008). Signals from regions along the gut-brain axis effect changes in the relative activity of two neuronal subpopulations. These signals include an orexigenic or appetite stimulating population co-expressing the neuropeptides neuropeptide Y (NPY) and agouti-related peptide (AgRP), and an anorexigenic or appetite suppressing population co-expressing the neuropeptides pro-opiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART) (Chaudhri et al. 2008). Changes in the relative release of these neuropeptides strongly influence appetite regulation, and ultimately, body weight control.

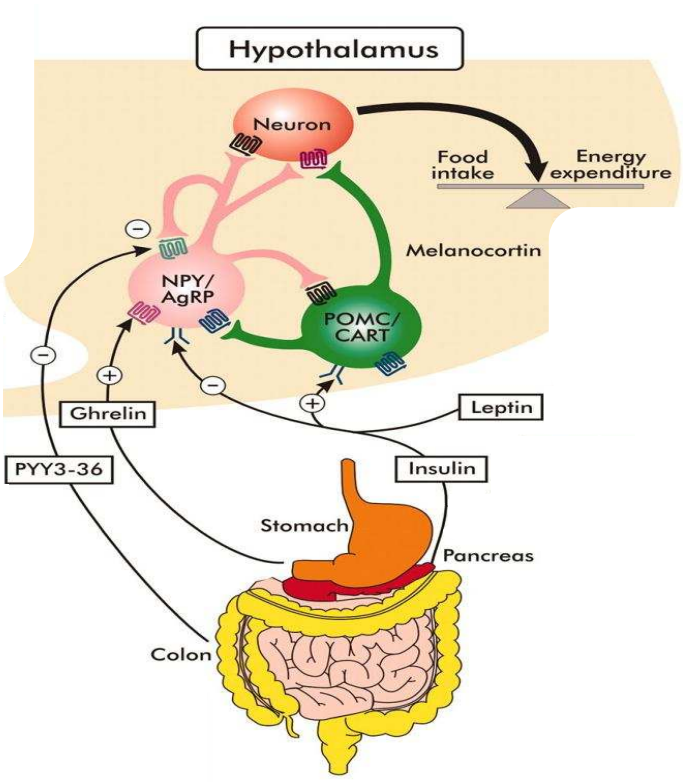


Figure 1-5: Central mechanisms in appetite regulation and body weight control: the gut/brain axis. Adapted from Geraedts et al. (2011). "Gastrointestinal targets to modulate satiety and food intake". Obes Rev 12(6): 470-477.

1.8.2. Gut hormones and neuropeptides

The GI tract represents the largest endocrine organ in the body and plays an important role in appetite regulation and body weight control as a source of regulatory peptide hormones (Hameed et al. 2009). The synthesis and release of gut hormones from a suite of endocrine cells occurs in response to nutrient intake and are thought to play a critical role in meal initiation and termination (Pournaras and Le Roux 2009). Along the gut-brain axis, neuropeptides such as NPY have also been documented to strongly influence the physiological control of body weight through appetite modulation (Valassi et al. 2008). In this thesis, the gut hormones ghrelin and peptide tyrosine-tyrosine (PYY), and the neuropeptide NPY, were primarily investigated and will be discussed. Their relation to the beneficial effects of weight loss will be outlined in Chapter 4.

1.8.2.1. Ghrelin and peptide tyrosine-tyrosine (PYY)

Ghrelin and peptide tyrosine-tyrosine (PYY) represent key gut hormones involved in the regulation of appetite and energy homeostasis, but have been shown to exert opposing effects (Kirchner et al. 2010).

Ghrelin is the only known orexigenic gut hormone and is produced primarily in the stomach (Kojima et al. 1999), and to a lesser degree, in regions of the small intestine (Date et al. 2000). Shortly after its discovery in 1999, the role of ghrelin in food intake regulation and body weight control was described (Tschop et al. 2000). Ghrelin has been demonstrated to be a potent food intake stimulator and meal initiator (Wren et al. 2001; Drazen et al. 2006). Appetite-stimulating effects in lean and obese

humans, including an approximately 30% increase in food intake at a buffet-style meal, have been documented (Murphy and Bloom 2004; Druce et al. 2005).

Peptide tyrosine-tyrosine (PYY) is an anorexigenic gut hormone produced and secreted mainly from the distal gut and ileum (Ekblad and Sundler 2002). Circulating levels of PYY are strongly influenced by meal composition and postprandial levels have been shown to become elevated within as little as one hour in humans (Adrian et al. 1985). Decreases in appetite and a resultant negative energy balance have been demonstrated in response to PYY in both lean and obese humans (Batterham et al. 2003). This effect has largely been attributed to interaction with its Y_2 receptor in the Arc, as targeted deletion of the Y_2 receptor has been shown to abolish the inhibitory effect of PYY on food intake (Batterham et al. 2002).

1.8.2.2. Neuropeptide Y (NPY)

Neuropeptide Y (NPY), whose expression predominates in the Arc, is the most potent activator of appetite in the CNS (Valassi et al. 2008). In addition, projections from NPY-expressing neurons to other CNS regions are capable of initiating appetite stimulating anabolic effects (Ramos et al. 2005). The anabolic effects of NPY have largely been attributed to interaction with its Y_1 and Y_5 receptors (Parker et al. 2002), two of the six NPY receptors which have been isolated and investigated to date. Modulation of NPY signaling in the Arc has been shown to influence energy balance in rats (Chao et al. 2011). Central injection of NPY into the hypothalamic Arc region has been shown to induce a marked feeding response and to stimulate food intake (Williams et al. 2004). Furthermore, chronic administration has been shown to lead to obesity (Zarjevski et al.

1993). Conversely, down regulation of NPY expression has been shown to reduce fat deposition and to improve high-fat diet-induced hyperphagia and obesity (Chao et al. 2011).

1.8.3. Effect of body weight on gut hormones and neuropeptides

Although the gut-brain axis represents an effective mechanism of body weight control through appetite regulation, long-term nutrient excess and resultant obesity have been shown to alter key components of this pathway, and in cases, to increase the difficulty by which the body can self-regulate the control of appetite and body weight (Roth and Reinehr 2010). The majority of the changes in gut hormones and other key components of the gut-brain axis seen in response to obesity have, however, been shown to be largely reversible in response to weight loss (Roth and Reinehr 2010).

Postprandial decreases in circulating ghrelin levels are attenuated, or even absent in the obese, suggestive of a role of ghrelin in the pathophysiology of obesity (le Roux et al. 2005). Conversely, circulating ghrelin levels have been reported as being significantly reduced in response to both diet- and bariatric surgery-induced weight loss (Cummings et al. 2002). Circulating PYY levels are often lower in the obese state, suggestive of a possible causative role of diminished PYY levels in the development of obesity (le Roux et al. 2006). Furthermore, postprandial increases in PYY levels typically seen in normal weight individuals are generally much lower, or even absent, in the obese state (Stock et al. 2005). In response to weight loss, however, significant increases in circulating PYY levels have been reported (Korner et al. 2005). Alterations in NPY levels have been shown to be associated with changes in energy balance (Sousa-Ferreira et al. 2011),

including evidence of a significant increase in NPY expression in response to obesity (Dryden et al. 1995) and decreased intake of high fat diets in response to central knockout of NPY (Sindelar et al. 2005).

1.9. Fatty acid oxidation and synthesis

The marked increase in the prevalence of obesity in recent years suggests that environmental and behavioural factors, such as increased intake of dietary fat and decreased physical activity, increase the likelihood of developing obesity, and have contributed substantially towards the classification of the disease as a worldwide epidemic (Bray et al. 2004; Greenberg and Obin 2006). Circulating fatty acids, which are well documented as a risk factor for several obesity-associated co-morbidities, are generally either oxidized for energy production or re-esterified from the circulation for storage as TG (Teusink et al. 2003). As such, promotion of fatty acid uptake and oxidation, rather than storage as TG, has become an increasingly attractive potential anti-obesity strategy (Niu et al. 2012).

In response to intake of dietary fat, oxidation of fatty acids has been shown to increase until its rate matches that of fat intake (Schrauwen et al. 1997). In the absence of a sufficiently compensatory increase in energy expenditure, however, the result has long been known to be an increase in fat mass (Flatt 1987). In metabolically healthy individuals, increased fat intake is matched with an increase in fatty acid oxidation (Boyle et al. 2011; Bergouignan et al. 2012). Conversely, a reduced capacity to oxidize fat predisposes to weight gain (Zurlo et al. 1990). In response to excessive dietary fat intake and obesity, the ability to adjust fat oxidation to fat intake is impaired (Schutz et al.

1989) and endogenous fatty acid oxidation capacity is reduced (Ceddia 2005). Further to the documented alterations in fatty acid oxidation in response to obesity, increased weight gain is associated with elevated hepatic lipogenesis, resulting in large part from stimulated expression of the lipogenic enzyme fatty acid synthase (FAS) (Diraison et al. 2002). Collectively, these obesity-associated abnormalities in fatty acid oxidation and synthesis alike have been reported to lead to increased circulating levels and accumulation of fatty acids and an increased risk of obesity-associated morbidity and mortality (Tanaka et al. 2005).

1.9.1. AMP-activated protein kinase (AMPK)-mediated fatty acid oxidation

Activation of metabolic pathways which promote fatty acid oxidation has been shown to prevent fatty acid accumulation in various tissues (Kahn et al. 2005). Conversely, a reduction in the capacity to oxidize fat predisposes to weight gain (Zurlo et al. 1990). It was once believed that transport of fatty acids across the plasma membrane for oxidation occurred by way of passive diffusion (Kiens and Roepstorff 2003). More recent evidence, however, has indicated that this process occurs in a protein-mediated fashion (Bonen et al. 2007). One such protein cascade that has been garnering significant attention as a mediator of cellular lipid homeostasis and fatty acid oxidation is driven by the heterotrimeric enzyme, AMP-activated protein kinase (AMPK) (Ceddia 2005; Niu et al. 2012) (Figure 1-6). When activated, AMPK has been shown to inhibit anabolic pathways and promote catabolism (Carling 2005). As such, activation of AMPK has been emerging as a potential strategy for the treatment of obesity (Kahn et al. 2005). Activated (phosphorylated) AMPK has been shown to phosphorylate (and inactivate) the

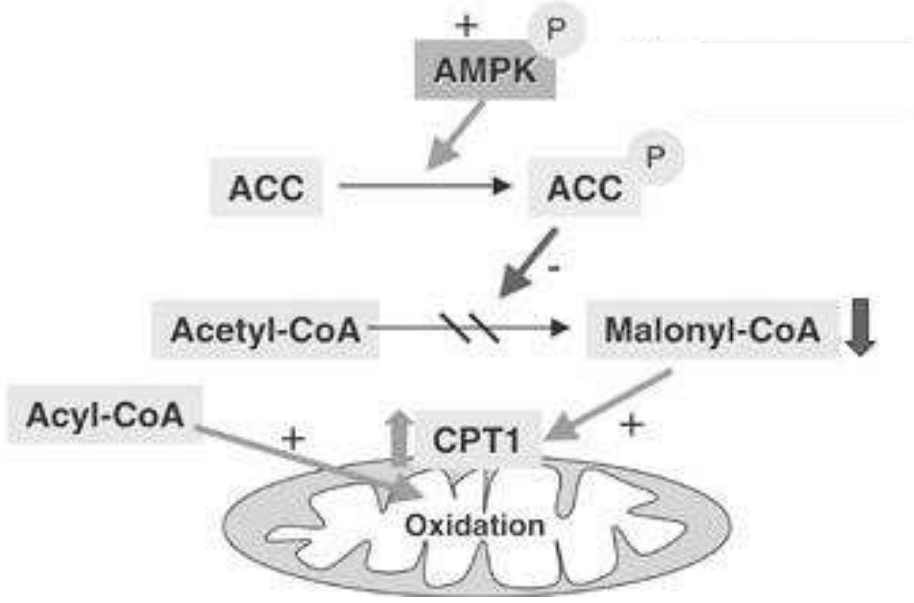


Figure 1-6: AMP-activated protein kinase-mediated fatty acid oxidation. Adapted from Hopkins et al. (2003). "AMP-activated protein kinase regulation of fatty acid oxidation". Biochem Soc Trans 31: 207-212.

downstream target, acetyl-CoA carboxylase (ACC), in effect blocking the production of malonyl-CoA (Harwood et al. 2003). Malonyl-CoA is an inhibitor of carnitine palmitoyltransferase-1 (CPT1), the rate-limiting enzyme responsible for the transport of fatty acids into the mitochondrion for beta-oxidation (McGarry 2002). Reduced malonyl-CoA production has been shown to result not only in a disinhibition of CPT1 activity, promoting the transfer of fatty acids into the mitochondrion for beta-oxidation, but also in a concomitant suppression of fatty acid synthesis (Kahn et al. 2005). By increasing fatty acid oxidation, the AMPK cascade has been reported to play an important role in protecting against obesity and several of its associated co-morbidities (Ceddia 2005). In line with this, however, the presence of excessive circulating or accumulated fatty acids has been reported to impair activation of the AMPK cascade (Taylor et al. 2005). Noteworthy is the recognition that obesity is associated with significantly elevated fatty acid levels, potentially compromising the oxidative response of the AMPK cascade (Roden 2004). A reduced capacity to oxidize fatty acids has been shown to be associated with excessive fatty acid accumulation, onset obesity, and an increased risk of obesity-associated morbidity and mortality (Tanaka et al. 2005; Holloway et al. 2009). As such, obese individuals stand to benefit from an enhancement in fatty acid oxidation capacity. Furthermore, an attenuation in the phosphorylation of AMPK (activation) and ACC (deactivation) have both been linked to obesity and several of its associated co-morbidities (Tanaka et al. 2005). Conversely, increased physical activity and decreased high-fat dietary intake have been shown to increase fatty acid oxidation, with resultant improvements in obesity and several of its associated co-morbidities (Bruce et al. 2006).

1.9.1.1. AMP-activated protein kinase (AMPK)

The heterotrimeric enzyme AMPK has been described as a key cellular energy sensor (King et al. 2006). Activation of AMPK occurs through phosphorylation of a threonine (threonine-172) residue within its catalytic α-subunit, either pharmacologically, by upstream kinases, or in response to increased cellular AMP levels (Kemp et al. 2003; Woods et al. 2003; King et al. 2006). AMPK is the physiological kinase of ACC, serves as an upstream regulator of CPT1, and is recognized as a key enzyme involved in the beta-oxidation of fatty acids and whole body lipid homeostasis (Munday 2002; Long and Zierath 2006; Ix and Sharma 2010). In addition, activation of AMPK has been shown to concomitantly inhibit fatty acid synthesis by way of decreasing FAS expression, as well as by way of its downstream effect on malonyl-CoA production (Woods et al. 2003; Guo et al. 2012).

Further to the noted beneficial effects of AMPK activation on fatty acid oxidation and synthesis, AMPK has also been emerging as a regulator of appetite at both the cell and whole-body levels (Kola 2008). In the brain, AMPK is widely expressed in areas linked to the control of food intake, such as the hypothalamus (Turnley et al. 1999). Therein, the activity of AMPK increases in the fasted state and decreases upon re-feeding (Minokoshi et al. 2004). Similar to the case in peripheral tissues, the downstream targets of AMPK in the hypothalamus are believed to include the ACC-malonyl-CoA-CPT1 pathway (Kola 2008).

1.9.1.2. Acetyl-CoA carboxylase (ACC)

In its active (dephosphorylated) form, ACC catalyzes the conversion of acetyl-CoA to malonyl-CoA for the endogenous synthesis of fatty acids by FAS (Wakil and Abu-Elheiga 2009). Conversely, in its inactive (phosphorylated) form, ACC has been shown to effectively decrease malonyl-CoA production, resulting in disinhibition of CPT1, increased shuttling of fatty acids into the mitochondrion, and a resultant increase in fatty acid oxidation potential (McGarry 2002).

1.9.1.3. Carnitine palmitoyltransferase-1 (CPT1)

Carnitine palmitoyltransferase-1 has been shown to play a major role in the regulation of fatty acid metabolism by orchestrating the rate-limiting step of fatty acid transport into the mitochondrion for beta-oxidation (McGarry 2002). Under conditions of elevated circulating fatty acid levels, such as in obesity, it has been shown that cells elicit a diminished capacity, through decreased activation of CPT1, to oxidize fatty acids (Pimenta et al. 2008). Conversely, exercise- and diet-induced weight loss have both been shown to increase CPT1 activity, and over expression of CPT1 in the presence of excessive circulating fatty acids has been shown to sufficiently enhance fatty acid oxidation (Bruce et al. 2006; Bruce et al. 2009).

1.9.2. Fatty acid synthase (FAS)

In addition to impaired fatty acid oxidation, obesity has been shown to be associated with dysregulated fatty acid synthesis (Diraison et al. 2002). Obesity-associated impairments in fatty acid synthesis have been reported to be due, in large part, to increased stimulation of FAS, a ubiquitously expressed lipogenic enzyme that

catalyzes the first committed step in the fatty acid biosynthesis pathway (Semenkovich 1997; Wang et al. 2004). In fact, dysregulated expression of FAS has been reported in obesity (Boizard et al. 1998) and increased FAS expression in adipose tissue of human subjects has been linked to excessive visceral fat accumulation (Berndt et al. 2007). Conversely, positive correlations between diet-induced weight loss and decreased expression of FAS have been documented (Tian et al. 2004).

1.10. Models of obesity

1.10.1. In vivo models of obesity

Variability has been shown to exist in the predisposition of certain individuals to gain weight in response to excessive dietary fat intake, while others appear to be resistant to the same effect (Cornier et al. 2009). Individuals prone to the development of obesity in response to high-fat diet feeding have been reported to have a decreased capacity to sense the energy value of high-fat dietary intake and/or have a blunted adaptive response to defend against weight gain (Schwartz et al. 2003). Conversely, individuals resistant to the development of obesity in response to high-fat dietary intake have been reported to have an increased capacity to more accurately sense energy balance and to respond adaptively to counteract the physiological tendency towards weight gain (Jackman et al. 2010).

For this thesis, the *in vivo* model chosen for investigation into the efficacy and subsequent mechanism of action of LWDH was the obesity-resistant (OR)/obesity-prone (OP)-CD rat model.

1.10.1.1. Obesity-resistant (OR)/obesity-prone (OP)-CD rat model

While most rats gain weight in response to high-fat diet feeding, some strains have been found to be more or less resistant to the same effect (Jackman et al. 2006). An *in vivo* model of obesity with a predisposition for the development of obesity, in large part due to environmental conditions such as increased dietary fat intake, was developed in 1997 (Levin et al. 1997). By using variability in weight gain in response to high-fat diet feeding as a selective breeding tool, the design for this model yielded two strains of Sprague-Dawley rats which have a similar phenotype when fed a low-fat diet, but diverge into either susceptible or resistant phenotypes when fed a high-fat diet (Levin et al. 1997). Similar to the condition of obesity in humans, these two phenotypes represent the crossroads of nature and nurture, that is, the interface between genetic background and environmental factors (Levin and Keesey 1998). As a result, the obesity-resistant (OR)/obesity-prone (OP)-CD rat model is now widely acknowledged as a representative model of the human condition of obesity (Jackman et al. 2006; Jackman et al. 2010).

Rats prone to high-fat diet-induced weight gain (OP) are generally larger than those resistant (OR) to the same effect, however, when fed a low-fat diet, body composition between the strains has been shown to remain very similar (Levin and Keesey 1998). Conversely, when fed a high-fat diet, OP rats become obese and develop several obesity-associated co-morbidities, including leptin and insulin resistance (Wang et al. 2001). Furthermore, OP rats have a greater tendency to store dietary fat, while OR rats have been shown to elicit a greater capacity to oxidize dietary fat (Jackman et al. 2006). Resistance to high-fat diet-induced weight gain in OR rats has been suggested to

be due, in large part, to an increase in energy expenditure and a shift towards the use of stored fat as an energy source (Jackman et al. 2010). Furthermore, resistance to obesity in these same rats has been attributed in large part to more tightly regulated control of food intake (Jackman et al. 2010). Overall, OR rats have been reported to sense and adapt to high-fat diet overfeeding more acutely and accurately than their OP counterparts (Jackman et al. 2010).

1.10.2. In vitro models of obesity

In order to investigate the corresponding mechanism of action responsible for the efficacy of potential anti-obesity products, extracts of the product are commonly tested using physiologically-relevant *in vitro* models (Novakofski 2004). For this thesis, and as will be presented in subsequent chapters, two potential body weight-lowering mechanisms of action of LWDH and its ethanol extract were investigated. These included appetite regulation and increased fatty acid oxidation/decreased fatty acid synthesis. Appetite regulation as a potential mechanism of action was tested by measuring alterations in key components of the gut-brain axis in response to LWDH and its ethanol extract. As a representative *in vitro* model of the GI tract, the human intestinal CaCo-2 cell line was employed. The neuroblastoma SH-SY5Y cell line was employed as a representative model of the CNS. Increased fatty acid oxidation/decreased fatty acid synthesis as a potential mechanism of action was tested by measuring alterations in key components of the AMPK-mediated fatty acid oxidation cascade and the lipogenic enzymes FAS in response to LWDH and its ethanol extract. As *in vitro* models of the liver and muscle tissue, respectively, human HepG2 hepatocytes and rat L6 skeletal muscle myotubes were employed.

1.10.2.1. Appetite regulation: CaCo-2 and SH-SY5Y cells

Harnessing of the physiological mechanism of appetite regulation has become an intensely investigated approach to body weight control and has been reported to involve a communication line from the gut to appetite regulating regions in the CNS (Gardiner et al. 2008). For this thesis, two gut hormones with opposing appetite-suppressing and appetite-stimulating roles (PYY and ghrelin, respectively), and one appetite-stimulating neuropeptide (NPY) along the gut-brain axis, were investigated.

Peptide tyrosine-tyrosine (PYY) is a potent appetite-suppressing gut hormone secreted mainly from the ileum (Ekblad and Sundler 2002). Ghrelin, the only known appetite-stimulating gut hormone, is produced mainly in the stomach, and to a lesser degree, in regions of the small intestine (Date et al. 2000). As such, the human intestinal CaCo-2 cell line, which has been reported to express several morphological and biochemical characteristics of small intestinal enterocytes (Sambuy et al. 2005), was chosen for use as a representative *in vitro* model. These cells grow in monolayer, have tight junctions between adjacent cells, and express small intestinal hydrolase enzyme activities more similar to fetal than adult ileal enterocytes (Sambuy et al. 2005). Furthermore, previous studies have employed CaCo-2 cells for investigating PYY and ghrelin expression (Sonoyama et al. 2000; Yeung et al. 2006) and its use has grown significantly in recent years (Sambuy et al. 2005). Neuropeptide Y, whose expression predominates in the hypothalamic Arc, is reportedly the most potent activator of appetite in the CNS, and modulation of its signaling has been shown to significantly influence energy balance (Valassi et al. 2008; Chao et al. 2011). The SH-SY5Y cell line has been widely used as a neuronal model since the 1980's, as these cells elicit several functional

and biochemical characteristics of neurons (Xie et al. 2010). Such characteristics include, among others, expression of neurofilament proteins, nerve growth factor receptors, and neuronal enzyme activity (Ciccarone et al. 1989). In addition, SH-SY5Y cells can proliferate in culture for long periods of time without contamination (Xie et al. 2010). The most commonly reported use of SH-SY5Y cells has been as an *in vitro* model of Parkinson's disease (PD) (Xie et al. 2010). However, there has also been precedent in the literature for the expression of NPY in SH-SY5Y cells (Wernersson et al. 1998; Magni et al. 2000). Undifferentiated SH-SY5Y cells are commonly used as a model in neurotoxicity and neuroprotection studies (Xie et al. 2010). Certain limitations of undifferentiated SH-SY5Y cells reported in the literature have included the notion that when undifferentiated, the cell line is continuously dividing and that there is a certain degree of unreliability as to whether the cell line will elicit the typically expected markers of mature neurons (Datki et al. 2003; Xie et al. 2010). To investigate the efficacy of LWDH and its ethanol extract on NPY expression in this thesis, the human neuroblastoma SH-SY5Y cell line was chosen for use as a representative *in vitro* model.

1.10.2.2. Fatty acid oxidation and synthesis: HepG2 cells and L6 myotubes

Obesity has been shown to be associated with impaired fatty acid oxidation, as well as dysregulated fatty acid synthesis (Diraison et al. 2002; Ceddia 2005). As such, promotion of fatty acid uptake and oxidation and/or inhibition of fatty acid synthesis, have become increasingly attractive potential anti-obesity strategies (Berndt et al. 2007; Niu et al. 2012). Correspondingly, activation of metabolic pathways which promote fatty acid oxidation, such as the AMPK-mediated fatty acid oxidation cascade and inhibition of the lipogenic enzyme FAS, have both been emerging as potential anti-

obesity targets (Diraison et al. 2002; Niu et al. 2012). As representative *in vitro* models of fatty acid metabolism in the liver and muscle tissue, human HepG2 hepatocytes (Lin et al. 2007; Guo et al. 2012) and rat L6 skeletal muscle myotubes (Watt et al. 2006; Kelly et al. 2010) were employed. HepG2 cells are a widely used human hepatocellular carcinoma cell line, which is highly differentiated and elicits several genotypic characteristics of normal liver cells (Sassa et al. 1987; Gerets et al. 2012). Their most common used has been in toxicity screening, but use of this cell line for investigating the effects of natural products on lipid metabolism have also been widely reported (Lin et al. 2007; Guo et al. 2012; Niu et al. 2012). One of the major limitations of the HepG2 cell line is its low metabolic capacity relative to primary hepatocytes (Xu et al. 2004), often times leading to limited detection of changes in gene expression patterns. The myogenic L6 cell line, developed in 1968 (Yaffe 1968), elicits several characteristics of skeletal muscle cells, such as contractility, acetylcholine receptors, and insulin-sensitive glucose transport capabilities (Elsner et al. 1998). Certain myogenic characteristics reported to be lacking in the L6 cell line include high glycogen levels and low glucose levels (Meyer 1989; Nakatani et al. 1997). A common use for the L6 cell line in recent years has been the investigation of treatment effects on FFA-induced insulin resistance (Chen et al. 2009) and themechanism of action through fatty acid oxidation (Watt et al. 2006; Kelly et al. 2010).

1.11. Project hypotheses and objectives

The present thesis consists of a series of *in vivo* and *in vitro* experiments designed to elucidate the anti-obesity efficacy and potential mechanism(s) of action of the traditional Chinese herbal formula, LWDH, and its ethanol extract.

The hypotheses were:

1. Liuwei Dihuang will lower body weight and improve body composition, blood lipids, and leptin and insulin resistance.

2. Liuwei Dihuang will improve biomarkers of inflammation, oxidative stress, and adiponectin production.

3. Liuwei Dihuang and its ethanol extract will lower body weight through regulation of appetite by modulating key components of the gut-brain axis.

4. Liuwei Dihuang and its ethanol extract will lower body weight through regulation of fatty acid oxidation and synthesis by modulating key components of the AMPK-driven fatty acid oxidation cascade and the lipogenic enzyme FAS.

These hypotheses were tested by setting the following four main objectives:

1. To determine the efficacy of LWDH on body weight and composition, food intake, blood lipids, and leptin and insulin resistance using a rat model of obesity.

2. To determine the efficacy of LWDH on inflammatory and oxidative stress biomarkers and adiponectin production using a rat model of obesity.

3. To determine the efficacy of LWDH and its ethanol extract on appetite regulation as a potential body weight-lowering mechanism of action *in vivo* and *in vitro.*

4. To determine the efficacy of LWDH and its ethanol extract on fatty acid oxidation and synthesis as a potential body weight-lowering mechanism of action *in vivo* and *in vitro.*

CHAPTER 2: EFFICACY OF LIUWEI DIHUANG PILL ON BODY WEIGHT AND COMPOSITION, FOOD INTAKE, BLOOD LIPIDS, AND LEPTIN AND INSULIN RESISTANCE IN OBESE RATS

A version of this chapter has been published with modification as:

Perry, B., J. Zhang, C. Sun, T. Saleh, Y. Wang (2012). "Liuwei dihuang lowers body weight and improves insulin and leptin sensitivity in obese rats." Evid Based Complement Alternat Med 2012: 847167.

2.1. Abstract

The present study investigated the efficacy of a Chinese herbal formula, Liuwei Dihuang (LWDH), as a potential natural weight-lowering product. Twelve obesity-resistant (OR-CD) and 48 obesity prone (OP-CD) rats were used. Following a 2 week acclimation period, the OP-CD rats were divided into 4 groups (n = 12 each). One group served as a positive control for obesity (OP), while the other 3 were challenged twice-daily by oral gavage with total daily dosages of 500, 1500, or 3500 mg/kg body weight (BW) LWDH, respectively, for 9 weeks. The OR-CD rats served as the normal control group and were gavaged with the vehicle. All rats were fed an AIN-93G diet modified to contain 60% energy from fat. The highest LWDH dose led to a significant reduction in body weight during the last 4 weeks of treatment. Food intake was also reduced from the second to the last week of treatment. The high LWDH dose lowered serum triglyceride (TG) and free fatty acid (FFA) levels, as well as body fat. Both the high and medium

doses lowered serum leptin and insulin levels. Liver function testing, assessed by measuring serum liver enzyme levels, revealed no adverse side effects of LWDH in rats under the current experimental conditions. The results of the present study suggest that LWDH has potential as a safe and effective preventive or therapeutic natural weight-lowering product.

2.2. Introduction

The obesity epidemic is no longer limited to the developed countries, but has long been known to be rapidly spreading to developing countries as well (Drewnowski and Popkin 1997; Popkin 1998). Associated with overweight and obesity is an increased risk for the development of a series of chronic diseases including, but not limited to, type 2 diabetes mellitus (T2DM), cardiovascular disease (CVD), dyslipidemias, and certain forms of cancer (Must et al. 1999; Mokdad et al. 2003). The rapidly increasing prevalence of obesity has also been reported to negatively influence the general quality of human life and to impose an enormous burden on the health care system (Fine et al. 1999; Lakdawalla et al. 2005). Thus, control of body weight has now become one of the top priorities in nutritional and medical research (Perry et al. 2012). Studies have shown that a reduction of as little as 5-10% body weight is sufficient to significantly improve many of the known obesity-associated co-morbidities (Fujioka et al. 1991; Goldstein 1992), such as dyslipidemia, increased circulating free fatty acid (FFA) levels, leptin and insulin resistance, and alterations in serum liver enzymes levels (Wood et al. 1988; Fujioka et al. 1991; Dattilo and Kris-Etherton 1992; Goldstein 1992).

Leptin and insulin play a critical role in energy homeostasis (Carlson et al. 2009). These hormones have been shown to effectively relay information about peripheral fat stores to the brain by acting on specific neurons in the hypothalamus and modulating eating behaviour and energy expenditure (Kaiyala et al. 1995; Abizaid and Horvath 2008). A common feature of obesity is the development of leptin and insulin resistance (Enriori et al. 2007; Weiss and Kaufman 2008), resulting in an elevation of the circulating levels of these hormones as a consequence of compensatory physiological responses to leptin and insulin insensitivity (Shulman 2000; Schwartz and Niswender 2004). With the onset of leptin and insulin resistance, several obesity-related blood biomarkers change, such as an increase in FFA and triglyceride (TG) levels in the circulation, as well as in the liver and muscle tissues (Stefan et al. 2002). Furthermore, decreased insulin sensitivity is strongly correlated with the onset of T2DM (Colagiuri 2010).

To date, lifestyle changes including dietary habits and exercise remain the primary intervention for weight loss or control (Stafford and Radley 2003; Caterson et al. 2012). Collectively, weight loss and weight maintenance by way of lifestyle changes have been reported to be associated with a reduced risk of developing the most serious obesity-associated co-morbidities, as well as a general improvement in the overall quality of life (Thompson et al. 1999; Caterson et al. 2012). However, mediocre long-term compliance has continued to significantly challenge its effectiveness (Klonoff and Greenway 2008). Few dietary supplements or drugs have been successful in the prevention or treatment of obesity (Klonoff and Greenway 2008). Furthermore, most available pharmacological treatment strategies have been reported to have limited long-term success, a plethora of safety concerns, and are also often accompanied by weight

regain upon cessation of treatment (Li and Cheung 2011; Kang and Park 2012). Only bariatric surgery has been reported to effectively lead to substantial weight loss accompanied by long-term sustainability (Murphy et al. 2006), but its high cost and rate of mortality have rendered it largely impractical as a viable anti-obesity intervention (Adams et al. 2007). As a result, there exists a strong demand to continue searching for safe and efficacious products to combat this emerging health epidemic.

Traditional Chinese medicine (TCM) and the use of herbs and herbal formulations have been considered to have potential as an information source and starting point for the development of anti-obesity products (Perry et al. 2012). The ancient TCM Liuwei Dihuang (LWDH), prepared from a basic formula of 6 Chinese herbs (*Radix Rehmannide Preparata*, RRP; *Rhizoma Dioscoreae*, RD; *Fructus Corni*, FC; *Cortex Moutan*, CM; *Rhizoma Alismatis*, RA; and *Poria*) (Zhao et al. 2007; Xie et al. 2008), is widely produced in China in accordance with the China Pharmacopoeia standard of quality control (Fu et al. 2009). Despite its use worldwide for general health promotion (Ye et al. 2009), little is known about its potential benefits in regard to overall energy homeostasis and weight management. Furthermore, aside from limited reports of its effect on reducing visceral fat deposition in rats and inhibiting rat preadipocyte differentiation (Xue et al. 2006; Xiao et al. 2007), little to no investigation into its potential anti-obesity efficacy and mechanism(s) of action has been documented.

The aim of the present study was to investigate the anti-obesity efficacy of LWDH by measuring body weight and composition, food intake, blood lipid profiles, and the hormones leptin and insulin using a rat model of obesity. In order to evaluate any potential adverse side effects of LWDH, liver function tests were conducted by

measuring serum levels of a series of liver enzymes, including aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) and, γ-glutamyltransferase (GGT).

2.3. Materials and methods

2.3.1. Animals and diet

Sixty male rats were purchased from Charles River Laboratories (Montréal, QC, Canada). Twelve of these animals were of the obesity-resistant strain (OR-CD) and 48 were of the obesity-prone strain (OP-CD). All animals were housed individually in cages in a temperature-controlled room with a 12 hour light:dark cycle and acclimated for 2 weeks with free access to regular rodent chow and water. The OR-CD rats served as the normal control group (OR) and the OP-CD rats were randomly divided into 4 groups (n = 12 each) prior to the commencement of treatment. Randomization of the OP-CD rats was accomplished by ordering the rats based on initial body weight from lightest to heaviest and assigning rats sequentially to 1 of 4 groups. These groups included an obesity control (OP) and 3 treatments (T1A, T1B, and T1C). The T1A, T1B, and T1C groups were treated for 9 weeks by twice-daily oral gavage with total daily dosages of 500, 1500, or 3500 mg/kg body weight (BW) of the concentrated LWDH pills (Wanxi Pharmaceuticals Co. Ltd., Henan, China) suspended in water, respectively. The OR and OP control groups received water via oral gavage as the control vehicle. Treatment administration was performed during the light cycle; once in the morning and again 6 hours later. Although rats are generally more active and thus exert more energy during the dark period (Zucker 1971), unpublished data from the Wang lab shows that LWDH enhances energy expenditure at a larger magnitude during the light period. Throughout the treatment

period, all rats were fed an AIN-93G diet (Research Diets Inc., New Brunswick, NJ, USA), modified to contain 60% energy from fat (lard:sunflower oil, 96:4 weight/weight; purchased locally) (Table 2-1). Body weight and food intake were recorded daily. Daily food intake was calculated by subtracting the total food plus dish weight from that of the previous day. At the end of the study, all animals were fasted overnight and anaesthetized using Isoflurane (Abraxis BioScience, Richmond Hill, ON, Canada). Blood samples were collected via left ventricular cardiac puncture, placed on ice, and allowed to clot. After centrifugation at 2,500 g at 4ºC for 15 minutes, serum was collected, aliquoted, and stored at -80°C until analysis of each biomarker was conducted. Testicular, perirenal, and visceral fat depots were excised and rinsed briefly in 1 X phosphate buffered saline (PBS; Sigma). The depots were then weighed, recorded, and the total body fat mass of the 3 portions was calculated. The animal use and experimental protocols were approved by the Joint Animal Care and Research Ethics Committee of the National Research Council Canada and the University of Prince Edward Island. The study was conducted in accordance with the guidelines of the Canadian Council on Animal Care.

Table 2-1: AIN-93G diet composition.

Ingredient	Amount (g)	% of diet (g)
Casein	266	19.73
Corn starch	159	11.79
Sucrose	72	5.34
Fat*	348	25.81
Lard (96% of Fat)	334	24.77
Sunflower oil (4% of Fat)	14	1.04
Cellulose	66	4.90
DL-methionine	4	0.30
Mineral mix†	46	3.41
Vitamin mix†	13	0.96
Choline bitartrate	3	0.22
Butylated hydroxytoluene	0.3	0.022
Cholesterol	20	1.48
Cholic acid	3	0.22
Total	1348.3	100

*Modified to contain 60% energy from fat (lard:sunflower oil; 96:4 w/w).
†Composition of mineral and vitamin mixes are presented in APPENDIX A.

2.3.2. Preparation and dose determination of LWDH pill

The LWDH concentrated pills used in the present study were manufactured by Henan Wanxi Pharmaceuticals Ltd. Co. (Nanyang, Henan, China) using the 6 previously described Chinese herbs at a composition of 160 g RRP, 80 g RD, 80 g FC, 60 g CM, 60 g RA, and 60 g Poria. To ensure homogeneity, the pills were produced in accordance with the China Pharmacopoeia standard of quality control (Fu et al. 2009). Following extraction with 95% ethanol, the residue of CM was mixed with all RRP, RA, and Poria, and part of FC (27 g), followed by extraction with hot water (twice, 2 hours each). The water extract was then concentrated to form a paste, followed by mixing with powdered RD, FC (53 g), and the ethanol extract of CM to form the final product. Based on the recommended daily dosage of 24 pills (approximately 4.5 g/day) in humans and the previously reported dosage of 2.4 g/kg/day in rats (Xue et al. 2005), 3 dosages of 500, 1500, and 3500 mg/kg BW were chosen for the present study. The daily stomach gavage capacity of the rats used in the present study was determined to be approximately 6 mL (University Veterinarian, personal communication). Thus, daily treatments were prepared in a vehicle volume of 6 mL, administered twice-daily (approximately 3 mL per gavage session), and adjusted based on daily body weight recordings.

2.3.3. Measurement of serum lipids

Lipid levels in the serum, including total cholesterol (T-C), TG, and HDL-cholesterol (HDL-C) were measured in duplicate using a TC Matrix Biochemistry Analyzer (Teco Diagnostics, Anaheim, CA, USA) and supplied reagents. Non-HDL-C levels were calculated by subtracting HDL-C from T-C, as previously reported (Wang et al. 2010).

2.3.4. Measurement of serum free fatty acids

Serum FFA levels were measured in duplicate using commercial kits (BioVision Research Products, Mountain View, CA, USA) and in accordance with the kit instructions. Standards were prepared at a series of concentrations and run in parallel with the samples. The levels of FFA in the samples were calculated in reference to the corresponding standard curves and expressed as mmol/L.

2.3.5. Measurement of serum leptin and insulin

Serum leptin and insulin levels were measured in duplicate using commercial enzyme-linked immunosorbent assay (ELISA) kits (Crystal Chem Inc., Downer's Grove, IL, USA) and in accordance with the kit instructions. Standards were prepared at a series of concentrations and run in parallel with the samples. The leptin and insulin levels in the samples were calculated in reference to the corresponding standard curves and expressed as ng/mL.

2.3.6. Measurement of serum liver enzymes

Liver enzyme levels in the serum, including AST, ALT, ALP, and GGT were measured in duplicate using enzymatic methods on a TC Matrix Biochemistry Analyzer (Teco Diagnostics) and supplied reagents. Levels are presented as units/L (U/L).

2.3.7. Statistical analyses

Data analyses were performed by one-way ANOVA using SAS 9.2 statistical software (SAS Institute, Cary, NC, USA). Body weight and accumulative food intake were calculated weekly and analyzed using ANOVA with repeated measures. Differences between treatment means were determined by pair-wise comparisons using the least squares means test, with $P < 0.05$ indicating statistical significance. Results are presented as mean values with their standard errors.

2.4. Results

2.4.1. Effect of LWDH pill on body weight in obese rats

Throughout the 9 week treatment period, rats in the T1C treatment group had lower body weights, compared to the obesity control OP rats (Figure 2-1). This effect reached statistical significance in weeks 6, 7, 8, and 9 ($P = 0.03$, 0.02, 0.01, and 0.01, respectively). This was further highlighted by a total body weight reduction of 8% compared to the OP rats, beginning at week 8, and persisting until the end of the study. Under the current experimental conditions, the T1B and T1C treatments did not elicit significant body weight-lowering effects.

2.4.2. Effect of LWDH pill on body fat composition in obese rats

To determine the effect of LWDH on body fat composition, testicular, perirenal, and visceral fat depots were excised and weighed at the time of sacrifice. After the 9 week treatment period, rats in the T1C treatment group showed significantly less fat mass in the testicular, perirenal, and visceral regions ($P = 0.01$, 0.04, and 0.04, respectively),

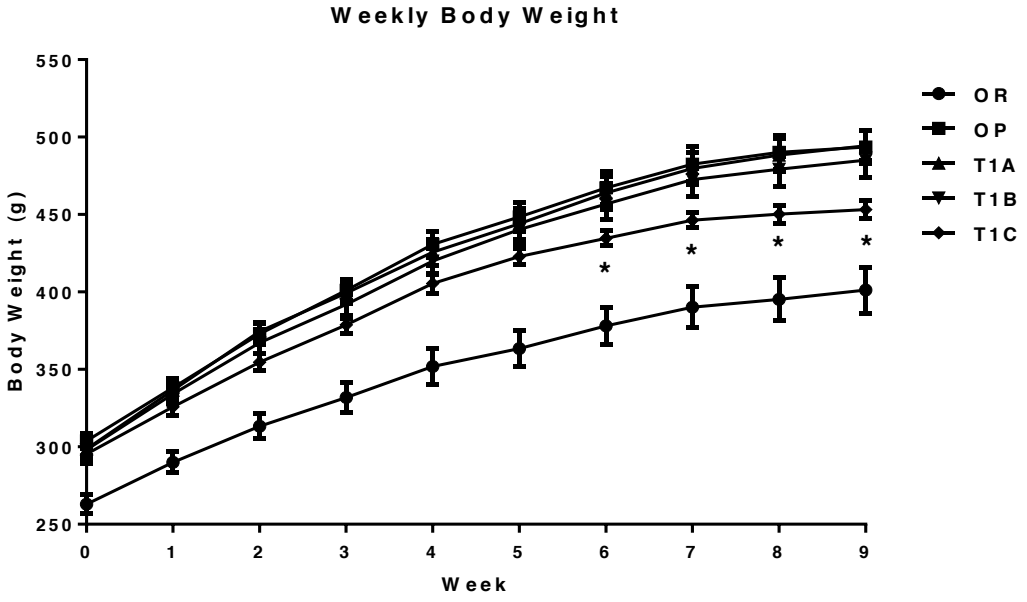


Figure 2-1: Effect of LWDH pill on body weight in obese rats. OR: obese-resistant normal control; OP: obese-prone positive control; T1A, T1B, and T1C represent the 3 OP groups treated twice a day by oral gavage with total daily doses of 500, 1500, or 3500 mg/kg BW LWDH suspended in water, respectively. All animals were fed a high-fat diet (AIN-93G diet modified to contain 60% energy from fat). Data were analyzed by one-way ANOVA with repeated measures. Differences between treatment means were determined by pair-wise comparisons using the least squares means test, with $P < 0.05$ indicating statistical significance. Data are expressed as mean values with their standard errors (n = 12). *Mean values were significantly different from OP control.

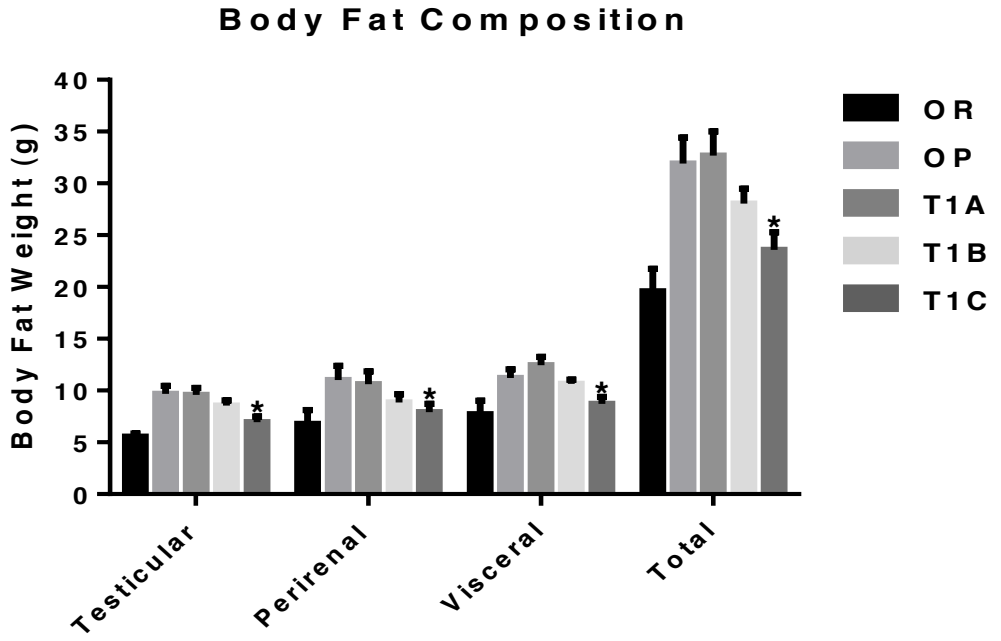


Figure 2-2: Effect of LWDH pill on body fat composition in obese rats. OR: obese-resistant normal control; OP: obese-prone positive control; T1A, T1B, and T1C represent the 3 OP groups treated twice a day by oral gavage with total daily doses of 500, 1500, or 3500 mg/kg BW LWDH suspended in water, respectively. All animals were fed a high-fat diet (AIN-93G diet modified to contain 60% energy from fat). Data were analyzed by one-way ANOVA. Differences between treatment means were determined by pair-wise comparisons using the least squares means test, with $P < 0.05$ indicating statistical significance. Data are expressed as mean values with their standard errors ($n = 12$). *Mean values were significantly different from OP control.

compared to the OP control rats (Figure 2-2). This observation was also noted in the total fat mass of the 3 regions ($P = 0.006$).

2.4.3. Effect of LWDH pill on food intake in obese rats

The effect of LWDH on weekly food intake is presented in Table 2-2. During the first week of treatment, food intake was not affected. During weeks 1 through 9, rats in the T1C treatment group had reduced food intake (P = 0.007, 0.0006, 0.002, 0.02, 0.0005, 0.0003, 0.02, 0.004, and 0.04, respectively), compared to the OP controls. Rats in the T1B treatment group had significantly reduced food intake during weeks 2 and 3 (P = 0.01 and 0.03, respectively), compared to the OP control. The T1A treatment did not show any significant effect. Feed efficiency data, calculated as weekly food intake divided by weekly weight gain, are presented in APPENDIX B.

2.4.4. Effect of LWDH pill on serum lipids in obese rats

At the end of the study, serum T-C levels in the T1C treatment group were reduced by 11% compared to the OP control but did not reach statistical significance (P = 0.13) due to large variations observed within the group (Figure 2-3). Serum TG levels in the T1C treatment group were significantly reduced by 28% (P = 0.01), compared to the OP control. The serum levels of HDL-C and non-HDL-C in each of the LWDH treatment groups did not differ when compared to the OP control.

Table 2-2: Effect of LWDH pill on weekly food intake in obese rats. OR: obese-resistant normal control; OP: obese-prone positive control; T1A, T1B, and T1C represent the 3 OP groups treated twice a day by oral gavage with total daily doses of 500, 1500, or 3500 mg/kg BW LWDH suspended in water, respectively.

	Control Groups		LWDH-treated Groups		
	OR	OP	T1A	T1B	T1C
Week 0 (g)	13.88 ± 0.79	14.57 ± 0.62	15.42 ± 0.62	15.39 ± 0.44	15.17 ± 0.40
Week 1 (g)	14.43 ± 0.61	15.34 ± 1.88	15.13 ± 0.36	14.14 ± 0.35	13.08 ± 0.61*
Week 2 (g)	12.91 ± 0.63	14.41 ± 0.55	15.07 ± 0.44	12.55 ± 0.54*	11.92 ± 0.52*
Week 3 (g)	13.35 ± 0.71	15.68 ± 0.89	15.03 ± 0.51	13.68 ± 0.62*	13.11 ± 0.36*
Week 4 (g)	13.67 ± 0.49	17.07 ± 0.38	16.31 ± 0.55	15.11 ± 0.89	15.93 ± 0.54*
Week 5 (g)	14.39 ± 0.64	14.36 ± 0.64	14.31 ± 0.73	13.75 ± 0.45	13.40 ± 0.42*
Week 6 (g)	14.76 ± 0.65	15.81 ± 0.78	15.78 ± 0.96	14.68 ± 0.85	13.58 ± 0.51*
Week 7 (g)	12.46 ± 0.86	14.16 ± 0.90	13.59 ± 0.52	13.14 ± 0.51	12.29 ± 0.51*
Week 8 (g)	12.86 ± 1.02	13.68 ± 0.65	14.48 ± 0.64	13.12 ± 0.76	13.25 ± 0.63*
Week 9 (g)	12.96 ± 0.84	14.42 ± 0.73	14.15 ± 0.58	15.10 ± 0.77	13.72 ± 0.65*

Data were analyzed by one-way ANOVA with repeated measures. Differences between treatment means were determined by pair-wise comparisons using the least squares means test, with $P < 0.05$ indicating statistical significance. Data are expressed as mean values with their standard errors ($n = 12$). *Mean values were significantly different from OP control.

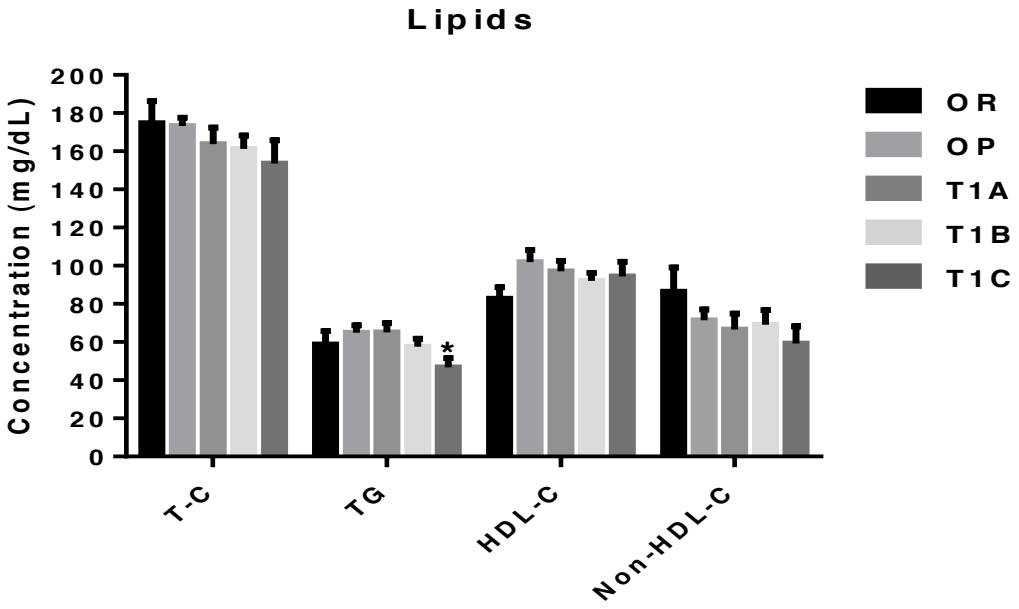


Figure 2-3: Effect of LWDH pill on serum lipids in obese rats. OR: obese-resistant normal control; OP: obese-prone positive control; T1A, T1B, and T1C represent the 3 OP groups treated twice a day by oral gavage with total daily doses of 500, 1500, or 3500 mg/kg BW LWDH suspended in water, respectively. All animals were fed a high-fat diet (AIN-93G diet modified to contain 60% energy from fat). Data were analyzed by one-way ANOVA. Differences between treatment means were determined by pair-wise comparisons using the least squares means test, with $P < 0.05$ indicating statistical significance. Data are expressed as mean values with their standard errors ($n = 12$). *Mean values were significantly different from OP control.

2.4.5. Effect of LWDH pill on serum FFA in obese rats

Compared to the OP control, rats in the T1C treatment group had significantly lower (P = 0.04) FFA levels (Figure 2-4). In addition, serum FFA levels in the T1B treatment group were reduced by 21% compared to the OP control, but did not reach statistical significance (P = 0.09).

2.4.6. Effect of LWDH pill on serum leptin and insulin in obese rats

Serum levels of leptin and insulin were measured at the end of the treatment period and are presented in Figure 2-5. Rats in both the T1B and T1C treatment groups had significantly lower serum leptin (P = 0.03 and 0.02, respectively) and insulin (P = 0.03 and 0.02, respectively) levels, compared to the OP control. The T1A treatment did not show any effect.

2.4.7. Effect of LWDH pill on serum liver enzymes in obese rats

To investigate the safety of the LWDH pill in the present study, serum levels of a suite of liver enzymes, including AST, ALT, ALP, and GGT were measured. The results indicated that none of the 3 LWDH doses altered the serum levels of AST, ALT, or GGT (Table 2-3). Notably, serum ALP levels were significantly reduced (P = 0.02) in the T1C treatment group, compared to the OP control.

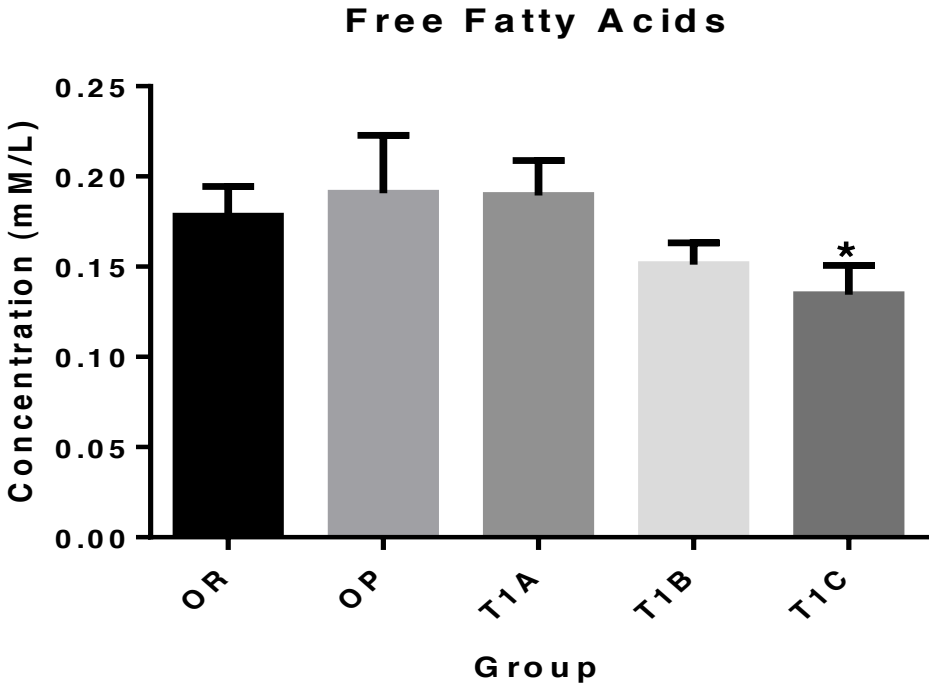


Figure 2-4: Effect of LWDH pill on serum free fatty acids in obese rats. OR: obese-resistant normal control; OP: obese-prone positive control; T1A, T1B, and T1C represent the 3 OP groups treated twice a day by oral gavage with total daily doses of 500, 1500, or 3500 mg/kg BW LWDH suspended in water, respectively. All animals were fed a high-fat diet (AIN-93G diet modified to contain 60% energy from fat). Data were analyzed by one-way ANOVA. Differences between treatment means were determined by pair-wise comparisons using the least squares means test, with $P < 0.05$ indicating statistical significance. Data are expressed as mean values with their standard errors ($n = 12$). *Mean values were significantly different from OP control.

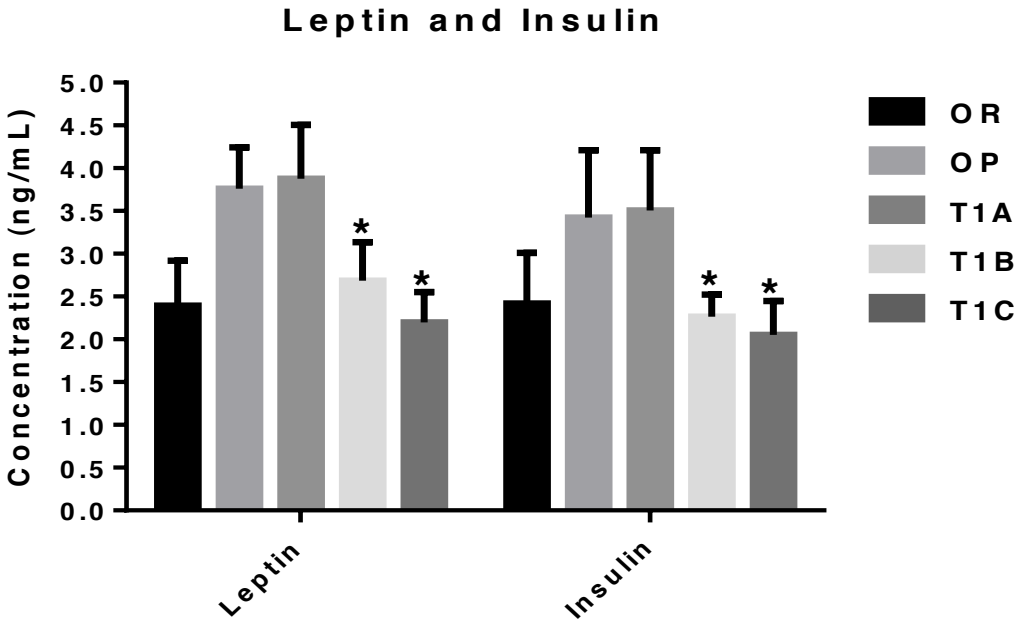


Figure 2-5: Effect of LWDH pill on serum leptin and insulin in obese rats. OR: obese-resistant normal control; OP: obese-prone positive control; T1A, T1B, and T1C represent the 3 OP groups treated twice a day by oral gavage with total daily doses of 500, 1500, or 3500 mg/kg BW LWDH suspended in water, respectively. All animals were fed a high-fat diet (AIN-93G diet modified to contain 60% energy from fat). Data were analyzed by one-way ANOVA. Differences between treatment means were determined by pair-wise comparisons using the least squares means test, with $P < 0.05$ indicating statistical significance. Data are expressed as mean values with their standard errors ($n = 12$). *Mean values were significantly different from OP control.

Table 2-3: Effect of LWDH pill on serum liver enzyme profile in obese rats. OR: obese-resistant normal control; OP: obese-prone positive control; T1A, T1B, and T1C represent the 3 OP groups treated twice a day by oral gavage with total daily doses of 500, 1500, or 3500 mg/kg BW LWDH suspended in water, respectively. U/L: units/L. All animals were fed a high-fat diet (AIN-93G diet modified to contain 60% energy from fat).

	Control Groups		LWDH-treated Groups		
	OR	OP	T1A	T1B	T1C
AST (U/L)	197.54 ± 25.14	178.79 ± 9.67	180.46 ± 12.09	187.25 ± 13.53	199.46 ± 12.77
ALT (U/L)	38.00 ± 1.83	46.04 ± 1.95	44.88 ± 1.51	41.88 ± 1.16	44.45 ± 2.06
ALP (U/L)	168.21 ± 13.15	295.75 ± 1.69	269.38 ± 6.16	271.29 ± 15.58	252.63 ± 11.68*
GGT (U/L)	8.71 ± 0.14	9.12 ± 0.25	8.83 ± 0.33	9.25 ± 0.23	8.77 ± 0.42

Data were analyzed by one-way ANOVA. Differences between treatment means were determined by pair-wise comparisons using the least squares means test, with $P < 0.05$ indicating statistical significance. Data are expressed as mean values with their standard errors ($n = 12$). *Mean were values significantly different from OP control.

2.5. Discussion and conclusions

The results of the present study have highlighted the potential anti-obesity efficacy and safety of the herbal formula, LWDH. When rats were treated with the high dose of LWDH, body weights were lowered significantly after 6 weeks of treatment and this effect persisted throughout the remainder of the treatment period. In line with the observed body weight reductions, testicular, perirenal, and visceral fat depots were also significantly lowered, demonstrating the beneficial effects of LWDH on body fat mass.

Overweight and obesity continue to grow as new threats to the general quality of human life and even small increases in weight have been documented to confer a significant negative impact on overall public health (Stein and Colditz 2004). Accumulating evidence, however, suggests that even a modest reduction in body weight of as little as 5-10% is sufficient to improve, or even abolish, several obesity-related complications (Goldstein 1992; Renehan et al. 2008). In addition to reducing body weight and improving body fat composition, high dose LWDH treatment also significantly reduced serum TG and FFA levels. Furthermore, both the medium and high doses of LWDH lowered serum leptin and insulin levels in a significant and dose-dependent manner. These parameters are all highly related to obesity and a reduction in any one is, in turn, beneficial to weight control and the mitigation of obesity-related complications (Boden 1998; Gao et al. 2007; Yuan et al. 2007).

In the present study, LWDH reduced body weight in obese rats by over 8% after 6 weeks of treatment when provided at a high dose of 3500 mg/kg/day. A similar and dose-dependent reduction was observed in regard to food intake, suggesting that the observed body weight reductions following LWDH treatment might, at least in part, be a result of decreased energy consumption. As such, Chapter 4 of this thesis will highlight investigations into appetite regulation as a potential body weight-lowering mechanism of action of the product.

A partially-refined concentrated pill form of LWDH was administered by oral gavage in the present study. In addition to its major bioactive components (Xie et al. 2008), the product has been reported to contain a high level of saccharides (Sangha et al. 2012), with very little to no bioactivity. Thus, the doses administered in the present study may appear to be higher than expected. The high dose LWDH treatment in the present study was, however, similar to that reported in a diabetes study in rats (Xue et al. 2005) and higher than the human dosage reported for general health promotion (Hu et al. 2005). The optimal dosage for weight management in humans has yet to be documented. The doses of LWDH used in the present study have potential to be significantly reduced when administered in the form of an extract or fraction and following dosage optimization. In addition, with respect to translation into the potential for anti-obesity use in humans, twice-daily (BID) oral dosing regimens have been shown to be equal (Insull et al. 1994), or superior (Ross et al. 2001), to once-daily regimes in terms of efficacy and safety.

Hyperlipidemia and increased circulating FFA levels are generally associated with obesity (Boden 2008). Furthermore, increased FFA levels in obesity are well

documented to be associated with the onset of insulin resistance (Boden 1997; Kahn et al. 2006), making them a likely culprit in the development of the most prevalent obesity-associated co-morbidity, T2DM (Wilding 2007). Metabolically, circulating FFA and TG are interrelated and their blood levels change in proportion to one another (Cusi 2010). In the present study, the high LWDH dose treatment significantly lowered blood TG and FFA levels, in line with the observed body weight reductions. Blood T-C levels were not significantly affected. It is well known that in addition to elevated circulating T-C levels, increased blood TG levels are an independent risk factor for the development of CVD (Yuan et al. 2007), the leading cause of mortality in developed countries (Kung et al. 2008). In the fasted state, circulating FFA arise primarily from within the adipose tissue, as a result of the hydrolysis of TG (Karpe et al. 2011). Following ingestion of a high-fat meal, circulating FFA are further produced, as a result of TG hydrolysis by adipose tissue lipoprotein lipase (Karpe et al. 2011). In addition, when FFA in the adipose tissue are taken up in adipocytes for storage, a portion escapes and joins the circulating FFA pool (Evans et al. 2002). This latter process, referred to as spillover, may in fact constitute upwards of fifty percent of the total circulating FFA pool in the postprandial period (McQuaid et al. 2011). Elevated circulating levels of TG and FFA as a result of increased adipose tissue mass and fat mobilization are commonly observed in obese individuals (Kahn et al. 2006; Karpe et al. 2011). The process of fatty acid mobilization from adipose tissue is normally suppressed by insulin (Karpe et al. 2011). In addition to its essential role in the control of body weight through suppression of food intake and stimulation of fatty acid oxidation, leptin enhances insulin sensitivity (Hedbacker et al. 2010; Koch et al. 2010). Thus, insulin and leptin play complementary roles in the regulation of nutrient

consumption, uptake, and oxidation (Morrison et al. 2009). Obesity is associated with resistance to the actions of both insulin and leptin , which can result in further elevations in circulating FFA (McLaughlin et al. 2001; Koch et al. 2010). For instance, in obesity, it has been reported that the mobilization of FFA from adipose tissue itself becomes insulin resistant, further increasing lipolysis (Karpe et al. 2011).An improvement in leptin and/or insulin sensitivity has been shown to stimulate fatty acid oxidation, correspondingly resulting in a reduction in circulating TG and FFA levels (Gil-Campos et al. 2004). The reductions in circulating TG and FFA levels observed in the present study might be a consequence of the observed body weight-lowering effects of LWDH, resulting in improved leptin and insulin sensitivity and thus fatty acid oxidation, in addition to reduced fat mobilization from adipose tissue and a consequential decrease in circulating FFA levels following weight loss. As such, Chapter 5 of this thesis will highlight investigations into increased fatty acid oxidation/decreased fatty acid synthesis as a potential body weight-lowering mechanism of action of the product.

Energy homeostasis requires a highly integrated control system involving neural, as well as both long- and short-term hormonal components (Stanley et al. 2005). Leptin and insulin, which are secreted in direct proportion to adipose tissue mass (Gil-Campos et al. 2004), have long been known to act as long-term signals of overall energy homeostasis (Kaiyala et al. 1995). In the obese state, an increase in adipose tissue mass leads to increased secretion of leptin and insulin into the circulation (Matsubara et al. 2002), often resulting in the development of hyperleptinemia and hyperinsulinemia, respectively (Fried et al. 2000; Matyskova et al. 2010). Normally, these hormones can relay information about peripheral fat stores to the brain and effectively modulate food

intake and energy expenditure (Frederich et al. 1995; Halaas et al. 1995) through inhibition and activation of anabolic and catabolic pathways, respectively (Fried et al. 2000). In the obese state, however, decreased sensitivity to the anti-obesity effects of these regulatory hormones often develops (Carlsson et al. 1997; Schwartz and Niswender 2004). In the present study, both the medium and high LWDH dose treatments significantly lowered serum leptin and insulin levels. The reduced levels of both hormones in the serum could be attributed to the observed body weight-lowering effect of LWDH, or by direct effects of LWDH on leptin and insulin sensitivity, independent of weight changes.

As LWDH is a herbal formula, it was also of interest to determine if any toxic effects may be linked to the administration of this specific product. Rats were monitored on a daily basis and no abnormal behaviours or activities were noted throughout the entire study period. In addition, liver function tests which are used routinely to detect the potential toxicity of a product (Nannipieri et al. 2005) were conducted. Analysis of 4 liver enzymes which are commonly used in the clinical toxicological testing did not reveal any toxic effects of LWDH under the current experimental conditions. Furthermore, serum levels of the analyzed liver enzymes have been reported as being indicative of a tendency towards the development of obesity (Messier et al. 2010). Decreased levels of these enzymes in obese individuals can reflect improved insulin sensitivity and reduced fat accumulation in the liver (Messier et al. 2010), whereas elevated levels reportedly represent an increased risk for the development of T2DM and CVD (Hanley et al. 2005; Thamer et al. 2005). The observed reductions in serum ALP levels in response to the high dose LWDH treatment might be indicative of improved insulin sensitivity, in line with

the reduction in blood insulin levels, in addition to the potential improvement of liver function following weight loss.

In conclusion, the present study has demonstrated the body weight-lowering efficacy of LWDH in obese rats, without any toxic effects observed during the treatment period by conducting liver function testing at the study's conclusion. Moreover, LWDH improved serum lipid profiles, including FFA levels, and lowered serum levels of leptin and insulin, which may be indicative of an improvement in leptin and insulin sensitivity. The results presented herein have shed light on the potential for the development of LWDH as a natural product for the management of obesity and several of its related complications. These findings could be related to the regulation of LWDH on appetite and/or fatty acid oxidation/synthesis, which have been further explored and reported in the subsequent chapters of this thesis.

CHAPTER 3: EFFICACY OF LIUWEI DIHUANG PILL ON INFLAMMATORY AND OXIDATIVE STRESS BIOMARKERS AND ADIPONECTIN PRODUCTION IN OBESE RATS

A modified version of this chapter has been submitted for publication as:

Perry, B., J. Zhang, T. Saleh, Y. Wang (2013). "Liuwei Dihuang, a traditional Chinese herbal formula, suppresses chronic inflammation and oxidative stress in obese rats". Submitted to Frontiers in Life Science.

3.1. Abstract

The present study was conducted to evaluate the efficacy of Liuwei Dihuang (LWDH) on obesity-associated biomarkers of inflammation, oxidative stress, and adiponectin production in obese rats, and to assess the suitability of the obese-prone CD (OP-CD) rat model for the study of these parameters. Following a 2 week acclimation period with free access to regular rodent chow and water, OP-CD rats were fed an AIN-93G diet modified to contain 60% energy from fat. Treatment occurred twice-daily by gavage feeding with doses of 500, 1500, or 3500 mg/kg body weight (BW) LWDH suspended in water (n = 12 rats per group). Twelve obese-resistant CD (OR-CD) rats served as the normal control. Biomarkers of inflammation, oxidative stress, and levels of the adipokine adiponectin were measured post-sacrifice. After 9 weeks of treatment, LWDH lowered serum C-reactive protein (CRP) and tumour necrosis factor-α (TNF-α) levels. Serum interleukin-6 (IL-6) levels were not affected. The activity of superoxide dismutase (SOD) in the liver was increased by all 3 doses of LWDH, while the levels of

reduced (GSH) and oxidized (GSSG) glutathione (and their ratio), and thiobarbituric acid reactive substances (TBARS) were unchanged. Serum adiponectin levels were increased by both the 500 and 1500 mg/kg BW LWDH doses. The results of the present study show, for the first time, the anti-inflammatory, anti-oxidative stress, and adiponectin-ameliorating efficacy of LWDH in obese-prone rats.

3.2. Introduction

Evidence accumulated over the last number of decades has demonstrated that obesity is strongly associated with chronic inflammation and systemic oxidative stress (Dandona et al. 2004). For example, the pro-inflammatory cytokine, tumour necrosis factor-α (TNF-α), has long been known to be constitutively expressed in adipocytes (Hotamisligil et al. 1993) and to be elevated in both the adipose tissue and blood of obese humans and some animal models of obesity (Kern et al. 1995; Dandona et al. 1998). The chronic inflammatory biomarker, C-reactive protein (CRP), and the acute phase pro-inflammatory cytokine, interleukin-6 (IL-6), have also been reported to be increased in obesity (Mohamed-Ali et al. 1997; Yudkin et al. 1999). In the obese state, adipose tissue has been reported to become a major source of circulating reactive oxygen species (ROS) (Furukawa et al. 2004), which at chronically high levels, can have deleterious effects on their targets, such as lipids, proteins, and DNA (Valko et al. 2007). In addition, increased circulating levels of ROS have been linked to the development of insulin resistance (Urakawa et al. 2003). Adipose tissue of obese subjects has also been shown to have suppressed activity of the antioxidant enzyme superoxide dismutase (SOD), decreased levels of glutathione (GSH), and increased levels of thiobarbituric acid reactive

substances (TBARS), a marker of lipid peroxidation and oxidative damage (Furukawa et al. 2004). In addition, oxidative stress and elevated circulating ROS from adipose tissue are involved in the dysregulated production of various adipokines, including the insulin-sensitizer adiponectin (Yamauchi et al. 2001). Collectively, increased levels of systemic biomarkers of inflammation and oxidative stress, along with dysregulated adiponectin production, represent some of the underlying causes of obesity and/or its associated co-morbidities, especially insulin resistance.

At the forefront of complementary and alternative treatment strategies against the ever increasing health threats of obesity and its associated co-morbidities, is traditional Chinese medicine (TCM) and the combination of different medicinal herbs into a single therapeutic formula (Day 2007; Yin et al. 2008). Such treatment strategies have been used as an integrative approach along with Western medicine, not only in China and other Asian countries, but increasingly more often in North America and Europe (Yin et al. 2008). Liuwei Dihuang (LWDH) is a classic Chinese herbal formula that has traditionally been used to restore functional insufficiency of the kidney, liver, and spleen (Ye et al. 2009). It is comprised of 6 Chinese herbs, including Radix Rehmanniae Praeparata (RRP; prepared root of *Rehmannial glutiosa*), Rhizoma Dioscoreae (RD; rhizome of *Dioscorea opposita*), Fructus Corni (FC; fruit of *Cornus officinalis*), Cortex Moutan (CM; root bark of *Paeonia suffruticosa*), Rhizoma Alismatis (RA; rhizome of *Alisma plantago-aquatica*), and Poria (scleorotia of *Poria cocos*) (Xie et al. 2008). This formula has recently been reported to have potential as a therapeutic natural product against obesity and to improve insulin sensitivity (Perry et al. 2012). In the present study, we report for the first time, the effect of LWDH on obesity-associated biomarkers of

inflammation and oxidative stress, and adiponectin production in obese-prone rats, and the possible causal link between obesity and associated insulin resistance.

3.3. Materials and methods

3.3.1. Animals and diet

The animals and experimental diet used in the present study were as previously reported in Chapter 2. Sixty male rats were purchased from Charles River Laboratories (Montréal, QC, Canada). Twelve of these animals were of the obesity-resistant strain (OR-CD) and 48 were of the obesity-prone strain (OP-CD). All animals were housed individually in cages in a temperature-controlled room with a 12 hour light:dark cycle and acclimated for 2 weeks with free access to regular rodent chow and water. The OR-CD rats served as the normal control group (OR) and the OP-CD rats were randomly divided into 4 groups (n = 12 each) prior to the commencement of treatment. Randomization of the OP-CD rats was accomplished by ordering the rats based on initial body weight from lightest to heaviest and assigning rats sequentially to 1 of 4 groups. These groups included an obesity control (OP) and 3 treatments (T1A, T1B, and T1C). The T1A, T1B, and T1C groups were treated for 9 weeks by twice-daily oral gavage with total daily dosages of 500, 1500, or 3500 mg/kg BW of the concentrated LWDH pills (Wanxi Pharmaceuticals Co. Ltd., Henan, China) suspended in water, respectively. The OR and OP control groups received water via oral gavage as the control vehicle. Treatment administration was performed during the light cycle; once in the morning and again 6 hours later. Although rats are generally more active and thus exert more energy during the dark period (Zucker 1971), unpublished data from the Wang lab shows that LWDH enhances energy expenditure at a larger magnitude during the light period.

Throughout the treatment period, all rats were fed an AIN-93G diet (Research Diets Inc., New Brunswick, NJ, USA), modified to contain 60% energy from fat (lard:sunflower oil, 96:4 w/w). Body weight and food intake were recorded on a daily basis. Daily food intake was calculated by subtracting the total food plus dish weight from that of the previous day. At the end of the study, all animals were anaesthetized using Isoflurane (Abraxis BioScience, Richmond Hill, ON, Canada) following an overnight fast. Blood samples were collected via left ventricular cardiac puncture, placed on ice, and allowed to clot. After centrifugation at 2,500 g at 4ºC for 15 minutes, serum was collected, aliquoted, and stored at -80°C until analysis of each biomarker was conducted. Liver tissue samples were excised and rinsed briefly in 1 X phosphate buffered saline (PBS; Sigma). Samples were then weighed, recorded, transferred to pre-labelled aluminum foil packets, and placed in liquid nitrogen for short-term storage. Post-sacrifice storage occurred at -80°C until analysis of each biomarker. The animal use and experimental protocols were approved by the Joint Animal Care and Research Ethics Committee of the National Research Council Canada and the University of Prince Edward Island. The study was conducted in accordance with the guidelines of the Canadian Council on Animal Care.

3.3.2. Preparation and dose determination of LWDH pill

The LWDH concentrated pills used in the present study were manufactured by Henan Wanxi Pharmaceuticals Ltd. Co. (Nanyang, Henan, China) using the 6 previously described Chinese herbs at a composition of 160 g RRP, 80 g RD, 80 g FC, 60 g CM, 60 g RA, and 60 g Poria. To ensure homogeneity, the pills were produced in accordance with the China Pharmacopoeia standard of quality control (Fu et al. 2009). Briefly, following extraction with 95% ethanol, the residue of CM was mixed with all RRP, RA,

and Poria, and part of FC (27 g), followed by extraction with hot water (twice, 2 hours each). The water extract was then concentrated to form a paste, followed by mixing with powdered RD, FC (53 g), and the ethanol extract of CM to form the final product. Based on the recommended daily dosage of 24 pills (approximately 4.5 g/day) in humans and the previously reported dosage of 2.4 g/kg/day in rats (Xue et al. 2005), 3 dosages of 500, 1500, and 3500 mg/kg BW were chosen for the present study. The daily stomach gavage capacity of the rats used in the present study was determined to be approximately 6 mL (University Veterinarian, personal communication). Thus, daily treatments were prepared in a vehicle volume of 6 mL, administered twice-daily (approximately 3 mL per gavage session), and adjusted based on daily body weight recordings.

3.3.3. Measurement of serum C-reactive protein (CRP)

Serum C-reactive protein (CRP) was measured in duplicate using a commercial enzyme-linked immunosorbent assay (ELISA) kit (Kamiya Biomedical Company, Seattle, WA, USA) in accordance with the kit instructions. Standards were prepared at a series of concentrations and run in parallel with the samples. The levels of CRP in the serum were calculated in reference to the corresponding standard curves and expressed as μg/mL.

3.3.4. Measurement of serum tumour necrosis factor-α (TNF-α)

Serum tumour necrosis factor-α (TNF-α) was measured in duplicate using a commercial ELISA kit (Hycult Biotech Inc., Plymouth Meeting, PA, USA) in accordance with the kit instructions. Standards were prepared at a series of concentrations and run in

parallel with the samples. The levels of TNF-α in the serum were calculated in reference to the corresponding standard curves and expressed as pg/mL.

3.3.5. Measurement of serum interleukin-6 (IL-6)

Serum interleukin-6 (IL-6) was measured in duplicate using a commercial ELISA kit (Bioscience, San Diego, CA, USA) in accordance with the kit instructions. Standards were prepared at a series of concentrations and run in parallel with the samples. The levels of IL-6 in the serum were calculated in reference to the corresponding standard curves and expressed as pg/mL.

3.3.6. Measurement of liver superoxide dismutase (SOD) activity

The activity of superoxide dismutase (SOD) in the liver was measured in duplicate using a commercial kit (Cayman Chemical) in accordance with the kit instructions. Frozen liver tissue was weighed and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (1:10; Sigma) was added. Following tissue homogenization using a PowerGen 125 homogenizer (Fisher Scientific, Ottawa, ON, Canada) and centrifugation at 10,000 g at 4ºC for 10 minutes, the supernatant was removed and stored at -80°C until the analyses were performed. Standards were prepared at a series of concentrations and run in parallel with the samples. The activity of SOD in the liver was calculated in reference to the corresponding standard curve and expressed as U/g tissue.

3.3.7. Measurement of liver reduced (GSH) and oxidized (GSSG) glutathione

The levels of reduced (GSH) and oxidized (GSSG) glutathione in the liver were measured in duplicate using commercial kits (Cayman Chemical) in accordance with the kit instructions. Frozen liver tissue was weighed and 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (1:10; Sigma) was added. Following tissue homogenization and centrifugation at 10,000 g at 4ºC for 10 minutes, the supernatant was removed and stored at -80°C until further analysis. Standards were prepared at a series of concentrations and run in parallel with the samples. The levels of GSH and GSSG in the liver were calculated in reference to the corresponding standard curves and expressed as μmol/g tissue. The reduced (GSH) to oxidized (GSSG) glutathione ratio was calculated as reported previously (Park et al. 2011).

3.3.8. Measurement of liver thiobarbituric acid reactive substances (TBARS)

The level of thiobarbituric acid reactive substances (TBARS) in the liver were measured in duplicate using a commercial kit (Cayman Chemical, Ann Arbor, MI, USA) in accordance with the kit instructions. Frozen liver tissue was weighed and radioimmunioprecipitation assay (RIPA) buffer (1:100; Sigma) was added. Following tissue homogenization and centrifugation at 10,000 g at 4ºC for 10 minutes, the supernatant was removed and stored at -80°C until further analysis. Standards were prepared at a series of concentrations and run in parallel with the samples. The levels of TBARS in the liver were calculated in reference to the corresponding standard curves and expressed as nmol/g tissue.

3.3.9. Measurement of serum adiponectin

Serum adiponectin was measured in duplicate using a commercial ELISA kit (Millipore Corporation, MA, USA) in accordance with the kit instructions. Standards were prepared at a series of concentrations and run in parallel with the samples. Serum adiponectin levels were quantitated in reference to the corresponding standard curves and expressed as ng/mL.

3.3.10. Statistical analyses

Data analyses were performed by one-way ANOVA using SAS 9.2 statistical software (SAS Institute, Cary, NC, USA). Differences between treatment means were determined by pair-wise comparisons using the least squares means test, with $P < 0.05$ indicating statistical significance. Results are presented as mean values with their standard errors.

3.4. Results

3.4.1. Effect of LWDH pill on serum CRP in obese rats

The effect of LWDH on serum CRP levels in obese rats was assessed after the 9 week treatment period and is presented in Table 3-1. Serum CRP was significantly reduced in rats treated with the 500, 1500, and 3500 mg/kg BW LWDH doses ($P = 0.04$, 0.005, and 0.0004, respectively), compared to the obese control. Furthermore, the reductions in CRP levels in response to LWDH treatment occurred in a dose-dependent manner (16.5, 25.6, and 34.3 %, respectively).

3.4.2. Effect of LWDH pill on serum TNF-α in obese rats

The effect of LWDH on serum TNF-α levels in obese rats is presented in Table 3-1. Compared to the obese control, serum levels of TNF-α were significantly reduced in rats treated with LWDH doses of 500 (55.9 %), 1500 (40.7 %), and 3500 (39.7 %) mg/kg BW (P = 0.009, 0.01, and 0.03, respectively).

3.4.3. Effect of LWDH pill on serum IL-6 in obese rats

The effect of LWDH on serum IL-6 levels in obese rats is presented in Table 3-1. Rats treated with the doses of 500, 1500, and 3500 mg/kg BW LWDH had reduced serum IL-6 levels but these changes were not statistically significant.

3.4.4. Effect of LWDH pill on liver SOD activity in obese rats

The effect of LWDH on liver SOD activity in obese rats was assessed after the 9 week treatment period and is presented in Table 3-2. The activity of SOD in the liver was significantly increased in rats treated with the LWDH doses of 500 (20.7 %), 1500 (16.7 %), and 3500 (13.9 %) mg/kg BW, compared to the obese control (P = 0.005, 0.02, and 0.04, respectively).

3.4.5. Effect of LWDH pill on liver GSH and GSSG in obese rats

The effect of LWDH on liver GSH and GSSG levels is presented in Table 3-2. Compared to the obese control, liver levels of GSH and GSSG (or their ratio) were unaffected by any of the LWDH doses under the current experimental conditions.

3.4.6. Effect of LWDH pill on liver TBARS in obese rats

Similar to the effect of LWDH on liver glutathione levels, liver levels of TBARS were unaffected by LWDH in the present study (Table 3-2).

3.4.7. Effect of LWDH pill on serum adiponectin in obese rats

The effect of LWDH on serum adiponetin levels in obese rats is presented in Figure 3-1. In response to treatment with the doses of 500 and 1500 mg/kg BW LWDH, serum adiponectin levels were significantly increased (P = 0.03 and 0.04, respectively), compared to the obese control. The serum adiponectin levels in rats treated with the 3500 mg/kg BW LWDH dose did not differ from the control (P = 0.08).

Table 3-1: Effect of LWDH pill on serum inflammatory biomarkers in obese rats. OR: obese-resistant rats; OP: obese-prone rats; T1A, T1B, and T1C represent the 3 groups of OP rats treated by twice-daily oral gavage with total daily dosages of 500, 1500, or 3500 mg/kg BW of LWDH suspended in water, respectively. CRP: C-reactive protein; TNF-α: tumour necrosis factor-α; IL-6: interleukin-6.

	OR	OP	T1A	T1B	T1C
CRP (μg/mL)	186.7 ± 14.1^{a}	166.7 ± 10.3^{a}	139.2 ± 8.9^{b}	124.1 ± 7.5^{b}	109.6 ± 4.9^{b}
TNF-α (pg/mL)	599.6 ± 89.4^{a}	507.0 ± 69.8^{a}	223.8 ± 29.8^{b}	300.6 ± 81.2^{b}	305.6 ± 40.2^{b}
IL-6 (pg/mL)	528.4 ± 36.6^{a}	444.4 ± 43.7^{b}	423.2 ± 34.8^{b}	410.9 ± 47.7^{b}	360.4 ± 52.7^{b}

Data are expressed as mean values with their standard errors ($n = 12$). Mean values not sharing a common superscript letter are significantly different ($P < 0.05$).

Table 3-2: Effect of LWDH pill on liver oxidative stress biomarkers in obese rats. OR: obese-resistant rats; OP: obese-prone rats; T1A, T1B, and T1C represent the 3 groups of OP rats treated by twice-daily oral gavage with total daily dosages of 500, 1500, or 3500 mg/kg BW of LWDH suspended in water, respectively. SOD: superoxide dismutase; GSH: reduced glutathione; GSSG: oxidized glutathione; GSH:GSSG: reduced:oxidized glutathione ratio; TBARS: thiobarbituric acid reactive substances.

	OR	OP	T1A	T1B	T1C
SOD (U/g tissue)	9347.1 ± 336.2^{a}	7675.2 ± 355.1^{b}	9262.4 ± 523.6^{a}	8960.1 ± 259.4^{a}	8745.3 ± 329.6^{a}
GSH (μmol/g tissue)	1.7 ± 0.1^{a}	1.6 ± 0.1^{a}	1.6 ± 0.1^{a}	1.5 ± 0.04^{a}	1.40 ± 0.08^{a}
GSSG (μmol/g tissue)	1.7 ± 0.1^{a}	1.5 ± 0.09^{a}	1.5 ± 0.1^{a}	1.4 ± 0.04^{a}	1.43 ± 0.08^{a}
GSH:GSSG	1.0 ± 0.05^{a}	1.0 ± 0.01^{a}	1.0 ± 0.006^{a}	1.0 ± 0.03^{a}	0.98 ± 0.01^{a}
TBARS (nmol/g tissue)	55.4 ± 2.5^{a}	58.0 ± 3.2^{a}	60.2 ± 2.7^{a}	58.8 ± 2.9^{a}	60.97 ± 2.1^{a}

Data are expressed as mean values with their standard errors ($n = 12$). Mean values not sharing a common superscript letter are significantly different ($P < 0.05$). Liver weights between groups did not differ.

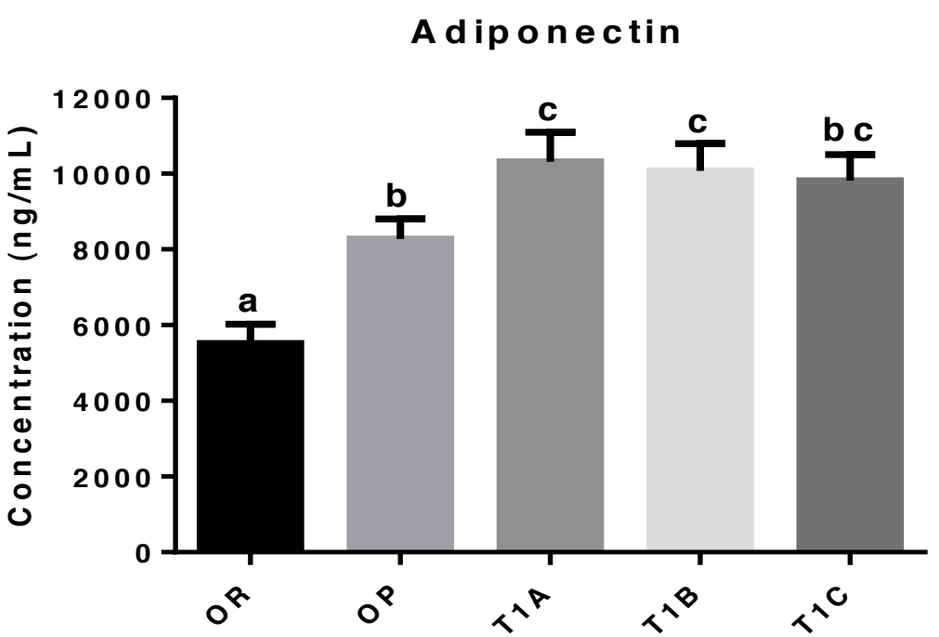


Figure 3-1: Effect of LWDH pill on serum adiponectin in obese rats. OR: obese-resistant rats; OP: obese-prone rats; T1A, T1B, and T1C represent the 3 groups of OP rats treated by twice-daily oral gavage with total daily dosages of 500, 1500, or 3500 mg/kg BW of LWDH suspended in water, respectively. Data are expressed as mean values with their standard errors (n = 12). Mean values not sharing a common superscript letter are significantly different ($P < 0.05$).

3.5. Discussion and conclusions

It is well known that obesity is associated with a state of chronic low-grade inflammation (O'Rourke 2009), which has been shown to impair insulin signalling and has provided a molecular link between inflammation and insulin resistance (Andersson et al. 2008; Fiorentino et al. 2010). The pro-inflammatory cytokine TNF-α is constitutively expressed in adipocytes and its expression has long been known to be upregulated in response to increased adiposity (Hotamisligil et al. 1993). As such, elevated circulating levels of TNF-α are often observed in obese humans and consistently in some obese animal models, including rats (Hotamisligil et al. 1993; Kern et al. 1995). Conversely, weight loss has been shown to lead to a reduction in circulating TNF-α levels (Kern et al. 1995). Similar to TNF-α, circulating levels of the systemic inflammatory biomarkers, CRP and IL-6, are elevated in response to overweight and obesity (Mora and Pessin 2002; Tsuriya et al. 2011). These inflammatory biomarkers have also been shown to be associated with the onset of insulin resistance (Pradhan et al. 2001; Lalli et al. 2008) and to decrease following weight loss (Dandona et al. 2004). In the present study, treatment with LWDH in obese rats resulted in significant reductions in circulating levels of CRP and TNF-α, but did not affect IL-6 levels. These observations are in line with the recently documented weight-lowering and insulin-sensitizing effects of LWDH in the same animal model (Perry et al. 2012) and may provide insight into the mechanisms of action of this herbal formula.

In addition to increased inflammation, oxidative stress is believed to be a possible culprit in the development of insulin resistance (Bloch-Damti and Bashan 2005), evidenced in part by reports of increased oxidative stress in insulin resistant humans

(Martinez-Hervas et al. 2008). In the obese state, adipose tissue is known to become a prominent source of circulating ROS (Furukawa et al. 2004). To protect against the potentially deleterious effects of ROS, cells are equipped with antioxidant systems, including SOD, which converts superoxide anions to hydrogen peroxide (H_2O_2) for rapid removal by detoxifying enzymes, such as glutathione peroxidase (GP_x) (Feillet-Coudray et al. 2009). Likewise, GSH can provide reduced ROS for GP_x-catalyzed H_2O_2 reductions (Feillet-Coudray et al. 2009). Often associated with increased adiposity is a suppression of SOD activity, resulting in a reduction in GSH levels and an increase in TBARS (Furukawa et al. 2004). Accordingly, reductions of adiposity are generally associated with an improvement in oxidative stress biomarkers and antioxidant defence systems (Heilbronn et al. 2006). In the present study, LWDH significantly increased liver SOD activity in obese rats. Despite observing a lack of effect on liver GSH, GSSG (or their ratio), or TBARS, similar effects have been reported in several tissues of high-fat diet-fed rats (Feillet-Coudray et al. 2009). Although not performed in the present study, analysis of GP_x may be useful in future studies, in order to elucidate whether the LWDH-mediated increase in liver SOD activity was accompanied by further downstream ROS detoxifying effects. Determining the starting source of ROS production may also be useful in further assessing the effect of LWDH on oxidative stress biomarkers. For instance, elevated ROS production from accumulated fat has been shown to lead to increased systemic oxidative stress and to mediate the obesity-associated development of insulin resistance.(Furukawa et al. 2004). Increased activity of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, which catalyzes the reduction of oxygen to generate superoxide radicals (Feillet-Coudray et al. 2009), is a major starting source of ROS (Valko et al. 2007) and its

expression has been shown to be increased in the adipose tissue of obese rodents (Furukawa et al. 2004). The present study was limited to the analysis of antioxidants (SOD and GSH) and oxidation products (TBARS) in the liver. Studies wherein analyses of SOD, GSH, TBARS, and other biomarkers were analyzed in multiple tissues, including blood, liver, muscle, and heart (Feillet-Coudray et al. 2009), have shown differential effects, depending on the tissue studied. Thus, in addition to the liver, the effect of LWDH on oxidative stress biomarkers may need to be evaluated in other tissues, such as various fat depots, particularly from the visceral region.

Obesity is known to be associated with dysregulated production of various adipokines (Furukawa et al. 2004), such as the insulin-sensitizer adiponectin (Yamauchi et al. 2001). It has been reported that adiponectin correlates inversely with adiposity, inflammation, oxidative stress, and insulin resistance (Arita et al. 1999; Furukawa et al. 2004). Despite gaps in the full elucidation of the mechanism by which increased adiposity leads to dysregulated production of adipokines, a decrease in the circulating level of adiponectin has been linked to the development of obesity-associated insulin resistance (Furukawa et al. 2004). The increase in adiponectin levels in obese rats following LWDH treatment in the present study may, in part, be attributable to the noted improvements in oxidative stress and/or suppression of chronic inflammation. This observation may also help explain our recent report of the insulin-sensitizing effect of LWDH in the same animal model (Perry et al. 2012), as discussed in Chapter 2 of this thesis.

In conclusion, the present study has demonstrated the beneficial effects of LWDH on biomarkers of inflammation and oxidative stress, and adiponectin production in obese rats. In addition, the present study has provided further evidence in support of the therapeutic potential of this herbal formula as a natural agent to prevent and/or treat insulin resistance.

CHAPTER 4: EFFICACY OF LIUWEI DIHUANG PILL AND EXTRACT ON APPETITE REGULATION *IN VIVO* AND *IN VITRO*

A version of this chapter has been published (as a review paper) with modification as:

Perry, B., Y. Wang (2012). "Appetite regulation and weight control: the role of gut hormones." Nutr Diabetes 2(1): 1-7.

4.1. Abstract

The present study investigated the appetite-regulating efficacy of Liuwei Dihuang (LWDH) as a potential body weight-lowering mechanism of action of the product. The herbal formula and its ethanol extract were tested on key components of the gut-brain axis *in vivo* and *in vitro*, respectively, by quantitative real-time polymerase chain reaction (qPCR). Ileal mRNA levels of peptide tyrosine-tyrosine (PYY) were unchanged and ghrelin mRNA levels were decreased in high dose LWDH-treated (3500 mg/kg BW; T1C) rats, compared to obese control (OP) rats. In the hypothalamus, neuropeptide Y (NPY) mRNA levels were unaffected in T1C rats, compared to OP rats. In CaCo-2 cells, representative of the gut, PYY mRNA levels were dose-dependently increased, reaching statistical significance in response to high concentration (100 μg/mL) LWDH ethanol extract treatment. Ghrelin mRNA levels were unchanged in the same cell line. In SH-SY5Y cells, representative of the CNS, NPY mRNA levels were unchanged. The results show that despite significant reductions in food intake as highlighted in Chapter 2 of this thesis, LWDH or its ethanol extract did not affect key components of the gut-brain axis at the gene expression level under the current experimental conditions. Thus, appetite

regulation does not appear to be responsible for the body weight-lowering mechanism of action of LWDH or its ethanol extract.

4.2. Introduction

The overwhelming increase in the prevalence of overweight and obesity in recent years has been associated with increased rates of morbidity and mortality, in addition to well documented personal, societal, and economic consequences (Hedley et al. 2004; Murphy and Bloom 2004). Even modest weight loss achieved through currently employed approaches can dramatically reduce the likelihood and severity of these consequences, yet bariatric surgery represents the only treatment offering sustainable weight loss results (Colquitt et al. 2009). Notably, post-bariatric surgery results have, in large part, been attributed to alterations in the physiology of circulating gut hormones and their appetite-regulating capabilities (Small and Bloom 2004). In tandem with the realization of the devastating global obesity epidemic and its associated co-morbidities in recent years, has been an increase in the recognition and understanding of the intricate interplay between gut hormones and the CNS (Woods and D'Alessio 2008), and the regulation of food intake and body weight through appetite modulation (Hameed et al. 2009). Several of these circulating appetite modulators, including ghrelin, the only known orexigenic gut hormone (Tschop et al. 2001), and a suite of anorexigenic gut hormones, such as peptide tyrosine-tyrosine (PYY), have been shown to influence appetite, and ultimately, body weight control (Batterham et al. 2003).

The gastrointestinal (GI) tract is the largest endocrine organ in the body and is believed to play an important appetite-regulating role as a source of various regulatory

peptide hormones (Chaudhri et al. 2008; Hameed et al. 2009). Postprandial satiety is believed to be regulated by a sensory system that communicates between the gut and appetite-regulating centers in the brain, with the hypothalamus being responsible for nutrient and energy sensing and corresponding adjustments in food intake (Murphy and Bloom 2004). In the gut, there exists a suite of endocrine cells which synthesize and release various hormones in response to nutrient and energy intake (Murphy and Bloom 2004) and it has been demonstrated that these hormones strongly influence appetite when administered at physiological levels (Batterham et al. 2002; Batterham et al. 2003).

Since its discovery in 1999 (Kojima et al. 1999), ghrelin has been proposed to act as a meal initiator, in large part due to its potent appetite-stimulating effects in free-feeding rats (Tschop et al. 2000). Although its signalling mechanisms remain to be completely understood, a particularly important role for the hypothalamic arcuate nucleus (Arc) has been suggested (Wren et al. 2001). Ghrelin has also been shown to stimulate appetite in both lean and obese humans (Wren et al. 2001; Druce et al. 2005), and administration (i.v.) in healthy individuals has been shown to increase appetite and food intake at a buffet-style meal by almost 30% (Murphy and Bloom 2004). The typically expected postprandial fall in circulating ghrelin levels is also attenuated, or even absent in the obese (English et al. 2002), suggestive of a role of ghrelin in the pathophysiology of obesity (le Roux et al. 2005).

Circulating levels of PYY are influenced by meal composition and calorie content, and postprandial levels have long been known to become elevated within as little as one hour in humans (Adrian et al. 1985; Murphy and Bloom 2004). In a trial consisting of both lean and obese humans, PYY administration (i.v.) was shown to lead to a decrease in

appetite and an almost 30% reduction in caloric intake in both groups (Batterham et al. 2003). With the anorexigenic capabilities of exogenous PYY being fully intact in the obese, resistance is not thought to manifest in the obese state. This has encouraged longer-term weight loss studies involving chronic administration. However, since circulating PYY levels are often lower in the obese state, it has been suggested that this characteristic may in fact play a causative role in the development of obesity (le Roux et al. 2006).

The regulation of feeding, energy intake and expenditure, and body weight is a homeostatic process (Wilding 2002). Information regarding general health is communicated predominantly via long-term hormonal signals, while meal initiation and termination are believed to be regulated via short-term signals, such as neural signals from the brain and hormonal signals from the gut (Kaiyala et al. 1995). The receipt and integration of these signals occur mainly in the hypothalamus and are largely regulated by the hypothalamic Arc (Murphy and Bloom 2004). Within the Arc, there exists two distinct populations of neurons responsible for appetite regulation, including the pro-opiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART) appetite-inhibiting co-expressing neurons, and the neuropeptide Y (NPY) and agouti-related peptide (AgRP) appetite-stimulating co-expressing neurons (Cone et al. 2001; Williams et al. 2001). Signals from the periphery result in changes in the relative activity of these two neuronal sub-populations and the release of their respective neuropeptides, subsequently influencing feeding behaviour and energy expenditure (Chaudhri et al. 2008). As a result of these collective reports, there has been increasing momentum aimed at turning this evidence-based knowledge into practical anti-obesity intervention.

The aim of the present study was to investigate the appetite-regulating efficacy of LWDH and its ethanol extract as a potential body weight-lowering mechanism of action of the product by measuring gene expression levels of key targets along the gut-brain axis.

4.3. Materials and methods

4.3.1. In vivo study

4.3.1.1. Animals and diet

The animals and experimental diet used in the present study were as described in Chapter 2. Sixty male rats were purchased from Charles River Laboratories (Montréal, QC, Canada). Twelve of these animals were of the obesity-resistant strain (OR-CD) and 48 were of the obesity-prone strain (OP-CD). All animals were housed individually in cages in a temperature-controlled room with a 12 hour light:dark cycle and acclimated for 2 weeks with free access to regular rodent chow and water. The OR-CD rats served as the normal control group (OR) and the OP-CD rats were randomly divided into 4 groups (n = 12 each) prior to the commencement of treatment. Randomization of the OP-CD rats was accomplished by ordering the rats based on initial body weight from lightest to heaviest and assigning rats sequentially to 1 of 4 groups. These groups included an obesity control (OP) and 3 treatments (T1A, T1B, and T1C). The T1A, T1B, and T1C groups were treated for 9 weeks by twice-daily oral gavage with total daily dosages of 500, 1500, or 3500 mg/kg body weight (BW) of LWDH (concentrated pills; Wanxi Pharmaceuticals Co. Ltd., Henan, China) suspended in water, respectively. The OR and OP control groups received water via oral gavage as the control vehicle. Treatment administration was performed during the light cycle; once in the morning and again 6 hours later. Although rats are generally more active and thus exert

more energy during the dark period (Zucker 1971), unpublished data from the Wang lab shows that LWDH enhances energy expenditure at a larger magnitude during the light period. Throughout the treatment period, all rats were fed an AIN-93G diet (Research Diets Inc., New Brunswick, NJ, USA) modified to contain 60% energy from fat (lard:sunflower oil, 96:4 w/w; purchased locally). Body weight and food intake were recorded on a daily basis. Daily food intake was calculated by subtracting the total food plus dish weight from that of the previous day. At the end of the study, all animals were fasted overnight and anaesthetized using Isoflurane (Abraxis BioScience, Richmond Hill, ON, Canada). Blood samples were collected via left ventricular cardiac puncture, placed on ice, and allowed to clot. After centrifugation at 2,500 g at 4ºC for 15 minutes, serum was collected, aliquoted, and stored at -80°C until analysis of each biomarker was performed. Ileal and hypothalamic tissue samples were excised and rinsed briefly in 1 X phosphate buffered saline (PBS; Sigma). Samples were then weighed, recorded, transferred to pre-labelled aluminum foil packets, and placed in liquid nitrogen for short-term storage. Post-sacrifice storage occurred at -80°C until analysis of each biomarker. The animal use and experimental protocols were approved by the Joint Animal Care and Research Ethics Committee of the National Research Council Canada and the University of Prince Edward Island. The study was conducted in accordance with the guidelines of the Canadian Council on Animal Care.

4.3.1.2. Preparation and dose determination of LWDH pill

The LWDH concentrated pills used in the present study were manufactured by Henan Wanxi Pharmaceuticals Ltd. Co. (Nanyang, Henan, China) using the 6 Chinese herbs described in Chapter 2 of this thesis, at a composition of 160 g RRP,

80 g RD, 80 g FC, 60 g CM, 60 g RA, and 60 g Poria. To ensure homogeneity, the pills were produced in accordance with the China Pharmacopoeia standard of quality control (Fu et al. 2009). Briefly, following extraction with 95% ethanol, the residue of CM was mixed with all RRP, RA, and Poria, and part of FC (27 g), followed by extraction with hot water (twice, 2 hours each). The water extract was then concentrated to form a paste, followed by mixing with powdered RD, FC (53 g), and the ethanol extract of CM to form the final product. Based on the recommended daily dosage of 24 pills (approximately 4.5 g/day) in humans and the previously reported dosage of 2.4 g/kg/day in rats (Xue et al. 2005), 3 dosages of 500, 1500, and 3500 mg/kg BW were chosen for the present study. The daily stomach gavage capacity of the rats used in the present study was determined to be approximately 6 mL (University Veterinarian, personal communication). Thus, daily treatments were prepared in a vehicle volume of 6 mL, administered twice-daily (approximately 3 mL per gavage session), and adjusted based on daily body weight recordings.

4.3.1.3. Rat ileal and hypothalamic tissue gene expression

In vivo expression of genes controlling appetite was conducted by way of quantitative real-time polymerase chain reaction (qPCR) using ileal and hypothalamic tissues of OP control and T1C rats.

4.3.1.3.1. Rat ileal and hypothalamic tissue RNA isolation

Isolation of RNA from rat ileal and hypothalamic tissues was performed by way of homogenization using the Trizol method, as previously reported (Rio et al. 2010). In brief, 1 mL Trizol reagent (Sigma-Aldrich Ltd., Oakville, ON, Canada) was

added per 100 mg frozen tissue to a 2 mL round-bottom tube (Sigma) and homogenized thoroughly using a PowerGen 125 homogenizer (Fisher Scientific, Ottawa, ON, Canada). Once homogenized, approximately 200 uL chloroform (Sigma; 1/5 the Trizol reagent volume) was added and the tubes were mixed vigorously using a bench top Mini Vortexer (Analytical Instruments, Golden Valley, MN, USA). Following incubation at room temperature and centrifugation at 10,000 g at 4ºC for 10 minutes using a bench top Mini Spin centrifuge (Eppendorf, Mississauga, ON, Canada), the aqueous phase was transferred to a clean 1.7 mL tube (Sigma). After addition of approximately 500 uL isopropanol (Sigma; 1/2 the Trizol reagent volume), vigorous vortexing, incubation at room temperature, and centrifugation at 12,000 g at 4ºC for 10 minutes, the isopropanol was removed. The remaining pelletized RNA was washed with 1 mL 75% EtOH (diluted in diethylpyrocarbonate (DEPC) H_2O; Merck, Darmstadt, Germany), vortexed briefly, and centrifuged at 10,000 g at 4ºC for 10 minutes. After removal of the 75% EtOH, the pelletized RNA was allowed to dry at room temperature. The dried pellet was then dissolved in DEPC H_2O. The samples were stored at -20ºC or processed immediately for measurement of concentration and purity.

4.3.1.3.2. Rat ileal and hypothalamic tissue RNA concentration and purity

Measurement of RNA concentration and purity from rat ileal and hypothalamic tissues was performed using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Rat ileal and hypothalamic pelletized RNA samples dissolved in DEPC H_2O were used at a volume of 2 µL for the measurement of RNA concentration (µg/mL) and purity (A_{260}/A_{280}). The cut-off for RNA purity was 1.8. Blank measurements were performed using 2 µL DEPC H_2O.

4.3.1.3.3. Synthesis of complementary DNA (cDNA)

For the synthesis of cDNA, enough RNA sample to obtain 2 µg cDNA, 4 µL qScript cDNA SuperMix (Quanta BioSciences Inc., Gaithersburg, MD, USA), and enough DEPC H_2O to reach a final volume of 20 µL were added to 200 µL reaction tubes (Sigma), vortexed, and centrifuged briefly. Reverse transcription to cDNA was performed using a thermocycler (Thermo Scientific) and under the following reaction conditions: 5 minutes at 25ºC, 30 minutes at 42ºC, 5 minutes at 85ºC, and a hold period at 4ºC. Following reverse transcription, the cDNA samples were diluted using DEPC H_2O. If not used immediately following reverse transcription and dilution, the samples were stored temporarily at -20ºC.

4.3.1.3.4. Quantitative real-time polymerase chain reaction (qPCR)

Quantitative real-time polymerase chain reaction was performed using 3 µL diluted cDNA and 12 µL qPCR MasterMix (7.5 µL SYBR Green SuperMix (Quanta BioSciences), 2.5 µL DEPC H_2O, 1 µL forward primer, and 1 µL reverse primer). The MasterMix was vortexed and centrifuged briefly prior to adding to the diluted cDNA samples. The qPCR reaction was carried out in strip PCR tubes (Roche Applied Science, Indianapolis, IN, USA) using a Corbett Rotor-Gene 6000 real-time rotary analyzer (Corbett Life Science, Concorde, NSW, Australia) under the following conditions, slightly modified from those previously reported (Reja et al. 2002): initial denature at 94ºC for 180 seconds followed by 40 cycles at 94ºC for 15 seconds, 57ºC for 20 seconds, and 72ºC for 25 seconds. Fold changes of target gene expression in response to treatment were calculated using the deltadelta CT method, as previously reported (Livak and

Schmittgen 2001), and normalized to the internal control (β actin). The primer sequences of the internal control and genes of interest (ghrelin, PYY, and NPY) were obtained from the literature (Lindqvist et al. 2008; Ferrer-Lorente et al. 2009), blasted to determine the sequence homology between species, and purchased from Sigma Genosys (Oakville, ON, Canada). The forward and reverse sequences for each primer are presented in APPENDIX A. Raw data are presented in APPENDIX C.

4.3.1. In vitro study

4.3.2.1. Preparation and concentration determination of LWDH ethanol extract

The ethanol extract of LWDH used in the present study was prepared from the standardized commercial concentrated LWDH pill described and used *in vivo* throughout this thesis, and as previously reported (Sangha et al. 2012): dry unpolished pills were milled and extracted twice with 95% ethanol (30 minutes each). Removal of the solvent under reduced pressure and drying at 70°C yielded the ethanol extract of LWDH used in the present study. Chemical profiling of the prepared ethanol extract of LWDH using various analytical techniques has recently been published (Sangha et al. 2012). The major compounds elucidated in the extract can be found in APPENDIX D. Data from the Wang lab currently under consideration for publication in Frontiers in Life Science show that in OP-CD rats fed a high-fat diet, the ethanol extract of LWDH reduces weight gain and visceral fat deposition. Furthermore, the extract decreases serum T-C, non-HDL-C, TG, FFA, and leptin levels.

Cell viability in response to various concentrations of the ethanol extract of LWDH in both CaCo-2 and SH-SY5Y cells was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Life Technologies, Inc.)

assay. A LWDH ethanol extract stock solution was made by dissolving the dried extract in dimethyl sulfoxide (DMSO; Sigma) and treatments ranging from 0-200 µg/mL were prepared in the cell type-specific complete medium. The concentration of DMSO in each treatment concentration was adjusted to 0.05%. Next, cells cultured to differentiation (CaCo-2) or confluency (SH-SY5Y) in 96-well plates (BD Biosciences, Mississauga, ON, Canada) were aspirated and treated with 200 µL of the prepared LWDH ethanol extract concentrations. After an overnight (24 hours) incubation at 37ºC, 20 µL of MTT stock solution (5 µg/mL) was added to each well and incubated for 4 hours at 37ºC. Following incubation, the wells were aspirated and 200 µL DMSO was added. The plate was then covered and shaken for 5 minutes on a plate rocker (Thermo Scientific) and the absorbance was read at 570 nm using a Varioskan Flash multimode reader (Thermo Scientific).

4.3.2.2. CaCo-2 and SH-SY5Y cell gene expression

In vitro expression of genes controlling appetite regulation was conducted by qPCR in CaCo-2 and SH-SY5Y cells.

4.3.2.2.1. Culture of CaCo-2 cells

Cells were cultured at 37ºC with 5% CO_2 in complete CaCo-2 growth media consisting of Eagle's Minimum Essential Medium (EMEM; American Type Culture Collection, Manassas, VA, USA) supplemented with 20% heat-inactivated fetal bovine serum (FBS; Life Technologies Inc., Burlington, ON, Canada) and 1% antibiotic (Penicillin/Streptomycin; Life Technologies, Inc.). Media was changed every 2-3 days.

4.3.2.2.2. Treatment of CaCo-2 cells with LWDH ethanol extract

Cells were grown in 75 cm^2 flasks (BD Biosciences). Upon reaching ~80% confluency, the media was removed and the cells were washed with 1 X PBS (Sigma). Trypsin (Cellgro, Manassas, VA, USA) was then added to detach the cells. When the cells were detached, fresh media was added to inactivate the trypsin. The flask contents were then transferred to 50 mL conical tubes (BD Biosciences). Following centrifugation at 10,000 g at 4ºC for 5 minutes, the media was removed and the cells were re-suspended in fresh media. The cells were counted using a hemocytometer (Hausser Scientific, Horsham, PA, USA) and diluted to a density of 3 x 10^5 cells/mL. An aliquot of 2 mL was added to each well to achieve the desired cell density of 6 x 10^5 cells/well. The media was changed every 2-3 days until the cells were differentiated (~11 days). Once differentiated, the cells were treated overnight (24 hours) at 37ºC with LWDH ethanol extract concentrations ranging from 0-200 µg/mL.

4.3.2.2.3. Culture of SH-SY5Y cells

Cells were cultured at 37ºC with 5% CO_2 in complete growth media consisting of Dulbecco's Modified Eagle Medium (DMEM; Life Technologies, Inc.) supplemented with 10% heat-inactivated FBS (Life Technologies) and 1% antibiotic (Life Technologies, Inc.). Media was changed every 2-3 days and cells were treated upon reaching 70-80% confluency.

4.3.2.2.4. Treatment of SH-SY5Y cells with LWDH ethanol extract

Cell culture conditions and treatment regimes were the same as for CaCo-2 cells, with minor modifications: SH-SY5Y cells were seeded (8 x 10^5 cells/well) and allowed to reach confluency prior to treatment.

4.3.2.2.5. CaCo-2 and SH-SY5Y cell RNA isolation

Isolation of RNA from CaCo-2 and SH-SY5Y cells was performed using the Trizol method (Rio et al. 2010). In brief, after aspiration of the media, 1 mL Trizol reagent (Sigma) was added to each well and the plates were kept at room temperature for 10 minutes. The well contents were then transferred to 1.7 mL tubes (Sigma), followed by addition of 200 µL chloroform (Sigma). The tubes were vortexed vigorously, incubated at room temperature for 10 minutes, and centrifuged at 12,000 g at 4ºC for 10 minutes. The top aqueous phase was then transferred to a new 1.7 mL tube (Sigma) and 500 µL isopropanol (Sigma) was added. The tubes were again vortexed vigorously, kept at room temperature for 10 minutes, and centrifuged at 12,000 g at 4ºC for 10 minutes. Following removal of the isopropanol, the RNA pellet was washed with 1 mL DEPC H_2O-diluted EtOH (75%), vortexed, and centrifuged at 10,000 g at 4ºC for 10 minutes. After removal of the 75% EtOH, the pelletized RNA was allowed to dry at room temperature and then re-dissolved in DEPC H_2O. The samples were stored at -20ºC or processed immediately for the measurement of concentration and purity.

4.3.2.2.6. CaCo-2 and SH-SY5Y cell RNA concentration and purity

Measurement of RNA concentration and purity from CaCo-2 and SH-SY5Y cells was performed as previously described for rat ileal and hypothalamic tissues.

4.3.2.2.7. Synthesis of complementary DNA (cDNA)

Synthesis of cDNA from CaCo-2 and SH-SY5Y RNA was performed as previously described for rat ileal and hypothalamic tissues.

4.3.2.2.8. Quantitative real-time polymerase chain reaction (qPCR)

Quantitative real-time polymerase chain reaction using CaCo-2 and SH-SY5Y cell cDNA was performed as previously described for rat ileal and hypothalamic tissues. The primer sequences (Sigma Genosys) used for the internal control (β actin) and genes of interest (ghrelin, PYY, and NPY) are presented in APPENDIX C. Raw data are presented in APPENDIX E.

4.3.3. Statistical analyses

Student's t-tests were used to compare results between the OP and T1C-treated groups in the *in vivo* analysis of the appetite-regulating efficacy of LWDH. The *in vitro* efficacy of the ethanol extract of LWDH was analysed statistically by one-way ANOVA. All analyses were conducted using SAS 9.2 statistical software (SAS Institute, Cary, NC, USA). Differences between treatment means were determined by pair-wise comparisons using the least squares means test, with $P < 0.05$ indicating statistical significance. Results are presented as mean values with their standard errors.

4.4. Results

4.4.1. In vivo study

4.4.1.1. mRNA expression of PYY and ghrelin in the ileum of obese rats

Expression of PYY and ghrelin genes in the ileal tissue of obese rats was assessed after a 9 week treatment period with LWDH. Compared to the OP control, the ileal gene expression of PYY was unchanged in response to the T1C treatment. Ghrelin

mRNA levels were significantly reduced (P = 0.001) by 59% in response to the T1C treatment (Figure 4-1).

4.4.1.2. mRNA expression of NPY in the hypothalamus of obese rats

The effect of LWDH on NPY gene expression in the hypothalamic tissue of obese rats is presented in Figure 4-2. The hypothalamic expression of NPY mRNA was unchanged in response to the T1C treatment, compared to the OP control.

4.4.2. In vitro study

4.4.2.1. Non-toxic concentration range of LWDH ethanol extract in CaCo-2 and SH-SY5Y cells

The results of the toxicity assessment of the LWDH ethanol extract in CaCo-2 and SH-SY5Y cells is presented in Figure 4-3 and Figure 4-4 a and b, respectively. In CaCo-2 cells, cell viability remained relatively stable following treatment with LWDH ethanol extract concentrations ranging from 0 to 100 µg/mL, but dropped to less than 70% at the concentration of 200 µg/mL. Thus, concentrations of 0, 25, 50, and 100 µg/mL were chosen for subsequent experiments using this cell line. In SH-SY5Y cells, a dramatic drop of cell viability to less than 65% of control was observed at the concentration of 50 ug/mL of the LWDH ethanol extract and continued to drop with the increase of concentrations. A further optimization was carried out and revealed that cell viability remained stable at a concentration range of 0-25 µg/mL. As such, concentrations of 0, 5, 10, and 25 µg/mL were chosen for subsequent experiments using this cell line.

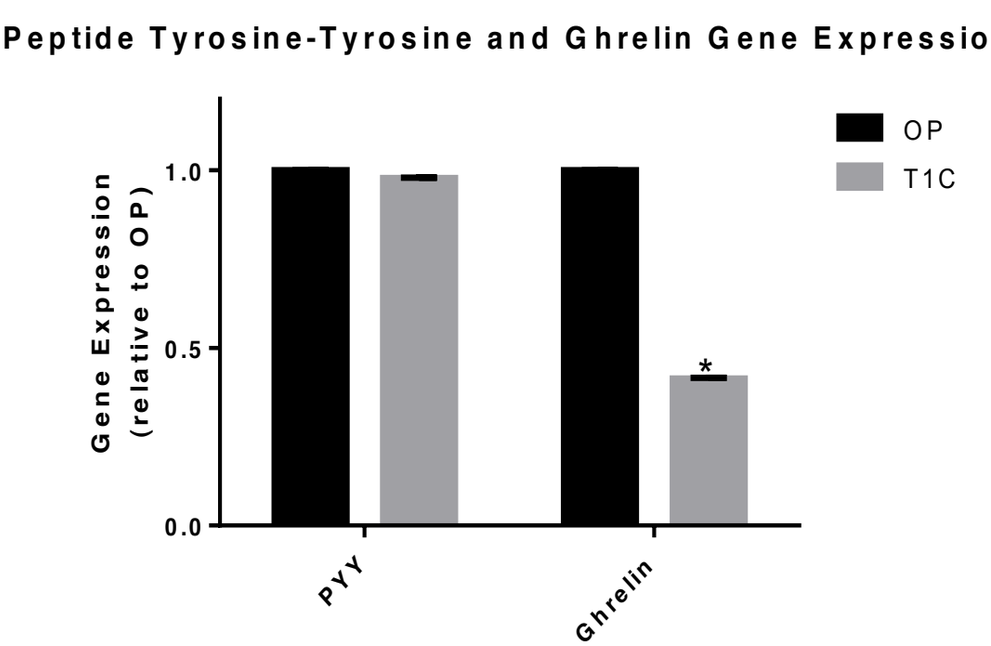


Figure 4-1: Effect of LWDH pill on ileal tissue PYY and ghrelin gene expression in obese rats. OP: obese-prone positive control; T1C: 3500 mg/kg BW LWDH-treated OP. All animals were fed a high-fat diet (AIN-93G diet modified to contain 60% energy from fat). Data were analyzed by student's t-test, with $P < 0.05$ indicating statistical significance. Data are expressed as mean values with their standard errors ($n = 12$). *Mean value of T1C was significantly different from OP control.

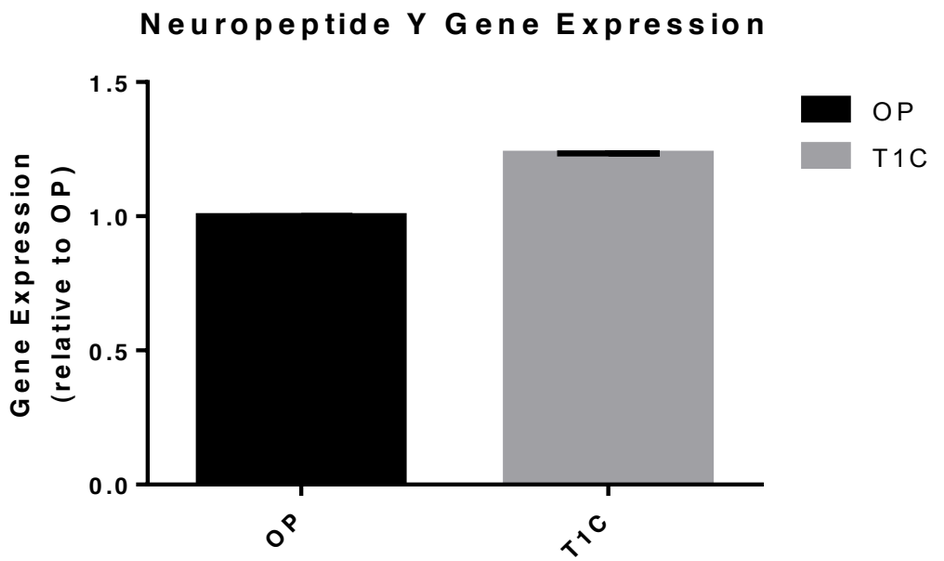


Figure 4-2: Effect of LWDH pill on hypothalamic tissue NPY gene expression in obese rats. OP: obese-prone positive control; T1C: 3500 mg/kg BW LWDH-treated OP. All animals were fed a high-fat diet (AIN-93G diet modified to contain 60% energy from fat). Data were analyzed by student's t-test, with $P < 0.05$ indicating statistical significance. Data are expressed as mean values with their standard errors ($n = 12$). *Mean value of T1C was significantly different from OP control.

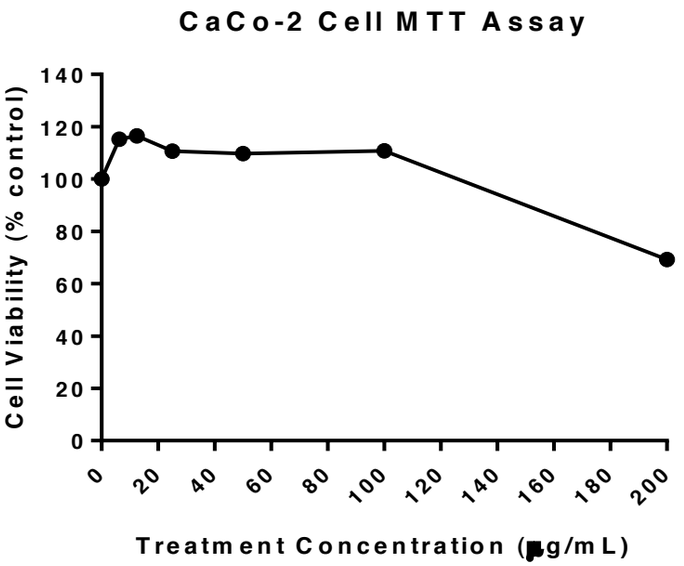
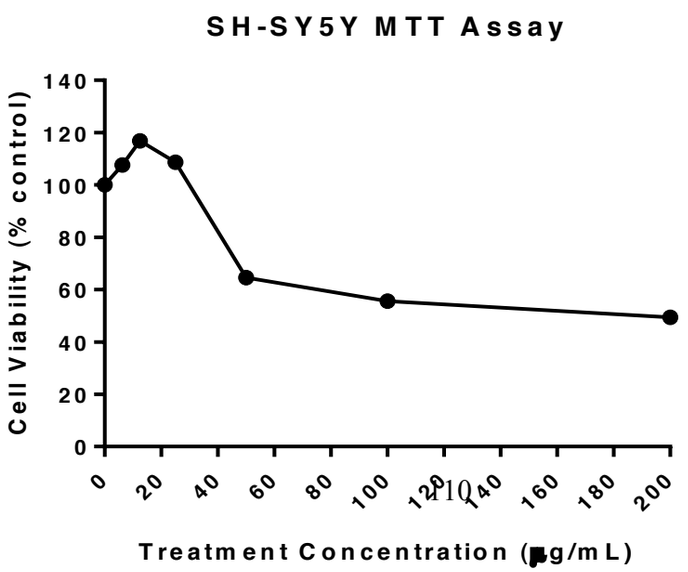


Figure 4-3: Non-toxic concentration determination of LWDH ethanol extract in CaCo-2 cells by MTT assay.



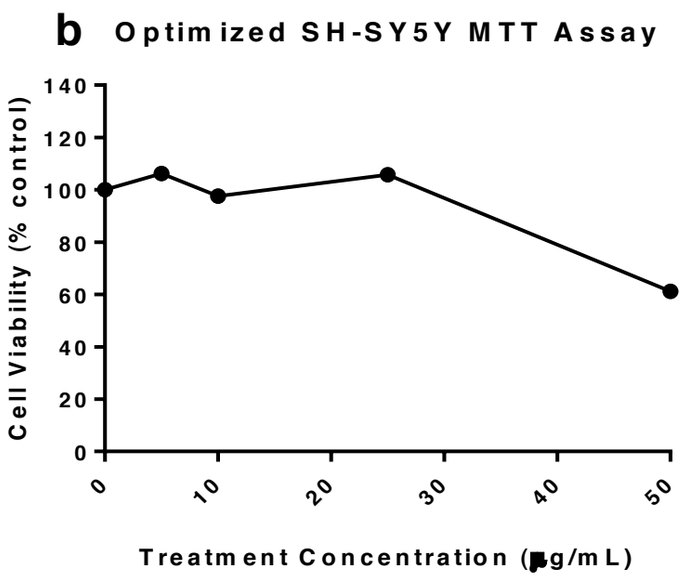


Figure 4-4: Non-toxic concentration determination of LWDH ethanol extract in SH-SY5Y cells by (a) MTT assay and (b) optimized MTT assay.

4.4.2.2. Gene expression of PYY and ghrelin in CaCo-2 cells

The effect of the LWDH ethanol extract on PYY and ghrelin mRNA levels in CaCo-2 cells is presented in Figure 4-5 and Figure 4-6, respectively. mRNA levels of PYY were increased in response to treatment with the LWDH ethanol extract and reached statistical significance ($P = 0.02$) at the concentration of 100 μg/mL. The LWDH ethanol extract did not show any significant effects on mRNA levels of ghrelin.

4.4.2.3. Gene expression of NPY in SH-SY5Y cells

The effect of the LWDH ethanol extract on NPY mRNA levels in SH-SY5Y cells is presented in Figure 4-7. The gene expression of NPY in SH-SY5Y cells was unchanged in response to the optimized concentrations of the LWDH ethanol extract, compared to control.

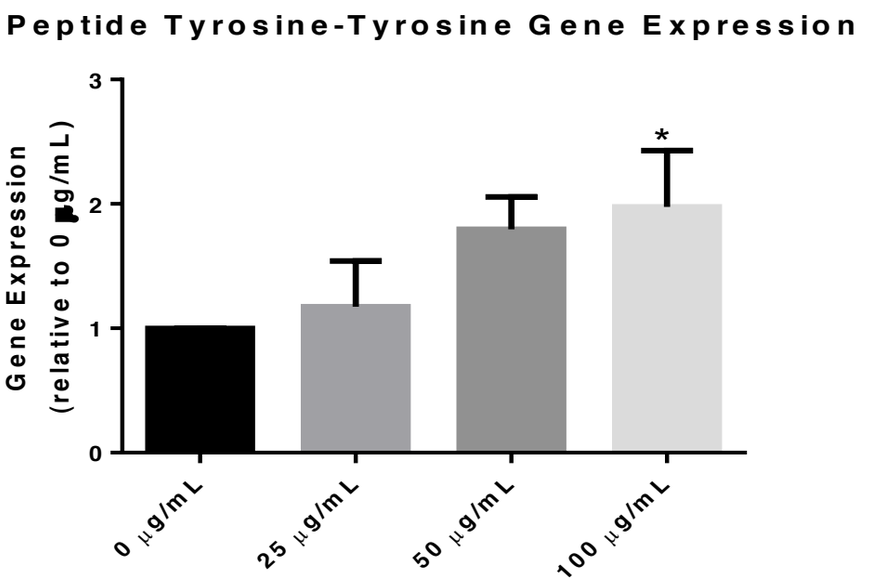


Figure 4-5: Effect of LWDH ethanol extract on PYY gene expression in CaCo-2 cells. Data were analyzed by one-way ANOVA. Differences between treatment means were determined by pair-wise comparisons using the least squares means test, with $P < 0.05$ indicating statistical significance. Data are expressed as mean values with their standard errors ($n = 3$ independent experiments). *Mean values significantly different from 0 ug/mL control.

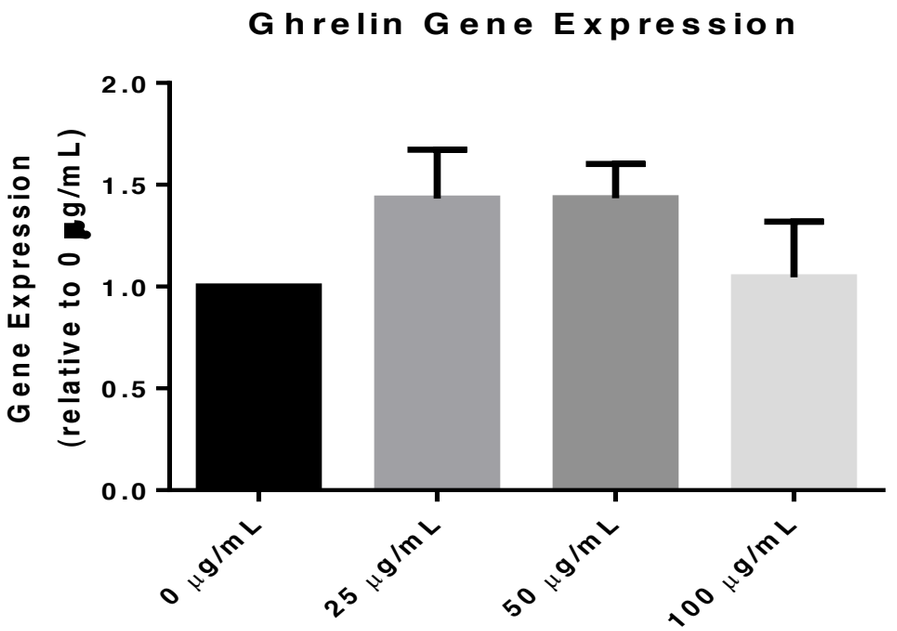


Figure 4-6: Effect of LWDH ethanol extract on ghrelin gene expression in CaCo-2 cells. Data were analyzed by one-way ANOVA. Differences between treatment means were determined by pair-wise comparisons using the least squares means test, with $P < 0.05$ indicating statistical significance. Data are expressed as mean values with their standard errors ($n = 3$ independent experiments).

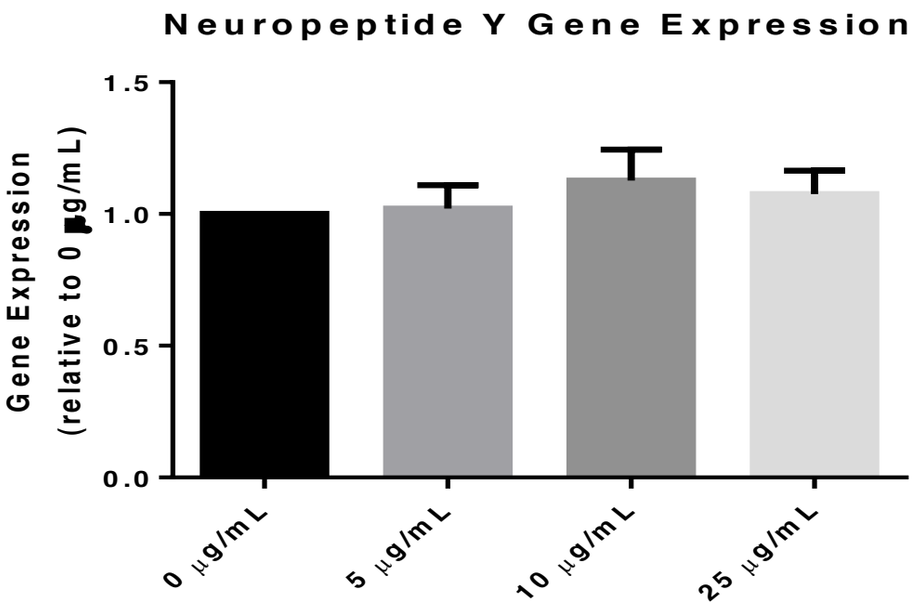


Figure 4-7: Effect of LWDH ethanol extract on NPY gene expression in SH-SY5Y cells. Data were analyzed by one-way ANOVA. Differences between treatment means were determined by pair-wise comparisons using the least squares means test, with $P < 0.05$ indicating statistical significance. Data are expressed as mean values with their standard errors ($n = 3$ independent experiments).

4.5. Discussion and conclusions

Harnessing of the physiological mechanism of appetite regulation has become an intensely investigated approach to body weight control and has been reported to involve a communication line from the GI tract to the CNS and resident centres of appetite regulation therein (Gardiner et al. 2008). The GI tract is the largest endocrine organ in the body (Hameed et al. 2009). Endocrine cells synthesize and release various hormones in response to nutrient and energy intake (Murphy and Bloom 2004) and it has been demonstrated that these hormones strongly influence appetite regulation (Batterham et al. 2003). In the hypothalamus, the Arc represents the primary CNS region involved in the control of food intake (Valassi et al. 2008). Signals from the GI tract cause changes in the relative activity of two neuronal subpopulations, resulting in either orexigenic (appetite-stimulating) or anorexigenic (appetite-suppressing) effects. As such, the line of communication between the GI tract and the CNS, referred to as the gut-brain axis, has been garnering significant recognition as a key component of a now well established model of appetite regulation (Chaudhri et al. 2008).

Ghrelin and PYY represent key gut hormones with opposing roles in the regulation of appetite and energy homeostasis (Kirchner et al. 2010). Stimulated release of PYY and diminished release of ghrelin have been investigated as potential therapeutic anti-obesity strategies (Cummings and Overduin 2007). Produced predominantly in the stomach (Kojima et al. 1999), ghrelin represents the only known orexigenic gut hormone (Hameed et al. 2009). Ghrelin binds to the growth hormone secretagogue (GHS) receptor, which is most highly expressed in the hypothalamus (Bailey et al. 2000). Subcutaneous injection of ghrelin has been shown to significantly induce appetite and increase food

intake (Druce et al. 2006). In obese subjects, fasting ghrelin levels have been shown to be lower compared to normal weight controls and to rise following diet-induced weight loss (Cummings et al. 2002). In contrast, circulating ghrelin levels have been reported to be markedly reduced post-bariatric surgery, thus potentially enhancing the body weight-lowering efficacy of the procedure (Cummings et al. 2002). Pharmacological blockage of ghrelin has been shown to result in decreases in food intake and body weight in rodents (Tschop et al. 2000), and GHS-deficient rodents have been shown to be resistant to diet-induced obesity (Wortley et al. 2005; Zigman et al. 2005). In diet-induced obese mice, GHS antagonists have been shown to cause significant reductions in body weight through fat loss, attributable to the centrally-mediated anorexigenic effects of GHS blockade (Esler et al. 2007). From a therapeutic utility standpoint, PYY has been shown to have anorexigenic effects in both normal weight and obese individuals, as inhibition of food intake in response to selective agonists and attenuation of this inhibitory effect in response to antagonists have both been shown (Lumb et al. 2007). Significant increases in circulating PYY levels have also been reported post-bariatric surgery (Korner et al. 2005), possibly contributing to the initial and long-term sustainment of weight loss attributed to the procedure. Development of a PYY nasal spray for thrice daily administration has been shown to result in modest weight reductions in humans (Gantz et al. 2007), however, side effects including nausea and vomiting were encountered during clinical trials. This, in addition to previously reported nausea and conditioned taste aversion in mice in response to food intake-lowering dosages (Halatchev and Cone 2005), has placed limitations on the utility of PYY as an anti-obesity agent.

In the present study, ghrelin gene expression in the ileum of obese rats was significantly reduced in response to high dose LWDH treatment. Ghrelin is expressed and secreted from the ileal region of the small intestine (Date et al. 2000; De Vriese and Delporte 2008) and mice with diminished ileal ghrelin expression are significantly leaner than controls (Duca et al. 2012). Furthermore, bypass of the ileal region in rats has been shown to lead to diminished levels of total ghrelin, with corresponding reductions in food intake and weight gain (Yan et al. 2011). Expression and secretion of ghrelin have, however, been shown to occur predominantly in the stomach (Kojima et al. 1999; Lim et al. 2011). At the time of sacrifice, the subsequent experimental plan had included investigation of circulating ghrelin levels from the serum. Unfortunately, the short half-life of endogenous ghrelin (Wynne et al. 2005) rendered expression levels undetectable. In addition, stomach tissue was not collected at the time of sacrifice for further biochemical or molecular analyses. As a result, although detectable, ghrelin expression levels in the ileal tissue may not have been sufficiently representative of the actual effect of LWDH on ghrelin gene expression. The intestinal CaCo-2 cell line has previously been used for investigating ghrelin and PYY gene expression levels (Sonoyama et al. 2000; Yeung et al. 2006). Thus, in the present study, CaCo-2 cells were used for *in vitro* investigation of the appetite-regulating efficacy of the ethanol extract of LWDH. Ileal tissue is also a well recognized site for investigating PYY gene expression (Bilchik et al. 1995; Duca et al. 2012). Ileal tissue gene expression levels of PYY in the present study were unaffected by LWDH treatment, and significantly increased in response to the 100 μg/mL dose of the LWDH ethanol extract. Nonetheless, the efficacy of LWDH and its ethanol extract on PYY gene expression under the current experimental conditions do not

appear to represent a potential body weight-lowering mechanism of action of the product. Development of more potent analogues, different administration routes or dosing regiments, or novel combinatorial approaches with other gut hormones may help unlock the future potential of PYY as an anti-obesity therapy.

The analyses of gene targets along the gut-brain axis in the present study were compared only between OP (positive control) and T1C (high dose LWDH-treated) rats. As a result, evaluation of the effect of obesity itself on ghrelin gene expression was not possible. Despite the beneficial ghrelin-lowering effects of high dose LWDH treatment in the ileal tissue of obese rats, ghrelin gene expression was unaffected in response to treatment with the ethanol extract of LWDH in CaCo-2 cells. Although as with PYY, alterations in ghrelin gene expression do not appear to represent a potential body weight-lowering mechanism of action of the product, further investigation into the ghrelin- or GHS-modulating effects of LWDH and its ethanol extract in obese rats, and especially in humans, may be warranted.

In the CNS, the hypothalamus is the key region involved in the regulation of appetite (Murphy and Bloom 2004). Gut hormone receptors are located on neuronal populations within the hypothalamic Arc (Small and Bloom 2004), which is also partially accessible to circulating appetite modulators due to its incomplete isolation from the blood-brain barrier (BBB) (Murphy and Bloom 2004). The orexigenic neuropeptide NPY is expressed in the hypothalamus (Fei et al. 2012) and modulation of NPY signaling has been shown to influence energy balance (Chao et al. 2011). Centrally-administered NPY has been shown to induce a marked feeding response and to stimulate food intake (Williams et al. 2004). Furthermore, chronic administration has been shown to lead to

obesity (Zarjevski et al. 1993). Conversely, knockdown of NPY expression has been shown to reduce fat deposition and to improve high-fat diet-induced hyperphagia and obesity (Chao et al. 2011). Furthermore, inhibition of hyperphagia and decreased intake of high-fat diets have been linked to central knockout of NPY (Sindelar et al. 2005). In the present study, NPY gene expression in both the hypothalamic tissue of LWDH-treated obese rats and in LWDH ethanol extract-treated SH-SY5Y cells was unchanged. Expression of NPY, however, predominates specifically in the hypothalamic Arc (Williams et al. 2001). At the time of sacrifice, the entire hypothalamic region of the brain was collected and processed for subsequent analyses. As such, other hypothalamic regions expressing NPY, albeit to a lesser degree, may have confounded the specificity of the results in the present study. In addition, although the SH-SY5Y cell line employed for investigating the efficacy of the LWDH ethanol extract on NPY gene expression is representative of the CNS, primary cell cultures using the specific Arc region of the hypothalamus may prove useful for more specific elucidation of the true effects in future investigations.

In contrast to some of the currently employed, relatively non-specific drug therapies, gut hormones act specifically on systems responsible for appetite control (Murphy et al. 2006). In addition, due to their natural physiological regulation of appetite, gut hormone-based therapies are reportedly less likely to cause adverse side effects compared to some of the currently approved drugs (Chaudhri et al. 2008). As such, gut hormone-based treatment approaches potentially offer a safer, more attractive alternative to combat the obesity epidemic. Although the gut-brain axis and a variety of hormone signalling pathways have been emerging as potentially viable anti-obesity targets, the

short half-life of many of the endogenous gut hormones must also be considered (Wynne et al. 2005; Murphy et al. 2006). Receptor agonists and alternative delivery routes have both been postulated to allow for circumvention of this unfortunate characteristic and to aid in the future development of gut hormone-based anti-obesity therapies (Chaudhri et al. 2008).

In conclusion, the present study has shed light on the therapeutic body weight-lowering utility of LWDH (decreased ghrelin gene expression) and its ethanol extract (increased PYY gene expression). However, concluding that appetite regulation represents a viable body weight-lowering mechanism of action of LWDH, or its ethanol extract, requires further investigation. Continued research into the potential to pharmacologically exploit endogenously occurring appetite-modulating gut hormones in an effort to regulate energy homeostasis is required. Nonetheless, there is strong evidence that obesity research and the development of weight loss or weight management products should, in large part, focus on the release and function of gut hormones, in connection to their association with neuropeptides and receptors in the CNS, in particular the hypothalamic Arc. Overall, the future development of safe and effective anti-obesity treatments through appetite modulation, in an effort to combat the rampant global rise in obesity, continues to have promising potential.

CHAPTER 5: EFFICACY OF LIUWEI DIHUANG PILL AND EXTRACT ON FATTY ACID OXIDATION AND SYNTHESIS *IN VIVO* AND *IN VITRO*

5.1 Abstract

The present study investigated the efficacy of Liuwei Dihuang (LWDH) on fatty acid oxidation and synthesis as a potential body weight-lowering mechanism of action of the product. In the liver, the high-fat diet-mediated down-regulation of acetyl-CoA carboxylase (ACC) and carnitine palmitoyltransferase-1 (CPT1) mRNA levels were not significantly reversed in response to treatment with the high dose of LWDH. In contrast, the high-fat diet-mediated up-regulation in the mRNA level of fatty acid synthase (FAS) was significantly reversed in response to the same treatment. Similar results were seen in the muscle, but the effects on CPT1 mRNA levels were statistically significant. The protein expression levels of phosphorylated ACC (pACC) and FAS in the liver were reversed in response to LWDH treatment, but the effects were not statistically significant. In the muscle, protein expression levels of phosphorylated AMP-activated protein kinase (pAMPK), pACC, CPT1, and FAS were significantly reversed. In HepG2 cells, no significant effects on target mRNA levels were observed in response to treatment with the ethanol extract of LWDH. In fatty acid (FA)-treated L6 myotubes, the mRNA level of AMPK was increased by the LWDH ethanol extract at the concentration of 100 μg/mL, while the mRNA level of FAS was decreased in response to the concentrations of 50 and 200 μg/mL. Significant increases in expression of pAMPK protein at 100 μg/mL, pACC protein at 200 μg/mL, and CPT1 protein at 100 and 200 μg/mL extract concentrations were observed. A significant decrease in FAS protein expression was seen in response to treatment with the 100 μg/mL LWDH ethanol extract concentration. Overall, up-

regulated fatty acid oxidation and/or down-regulated fatty acid synthesis appear to be responsible, at least in part, for the observed body weight-lowering effect of LWDH.

5.2. Introduction

Circulating free fatty acids (FFA), which are interrelated with obesity and are well documented as a risk factor for several obesity-associated co-morbidities, are generally either oxidized for energy production or re-esterified from the circulation for storage as triglycerides (TG) (Teusink et al. 2003). In response to dietary fat intake, oxidation of fatty acids increases until its rate matches that of intake, in order to maintain a constant body weight (Schrauwen et al. 1997). However, in the absence of a compensatory increase in energy expenditure, such as fatty acid oxidation, the result is an increase in fat mass (Flatt 1987; Kim et al. 2000). In metabolically-healthy individuals, increased fat intake is matched with increased fatty acid oxidation capacity (Bergouignan et al. 2012). Furthermore, a reduced capacity to oxidize fatty acids has been shown to predispose to weight gain (Zurlo et al. 1990). In addition to alterations in fatty acid oxidation capacity in obesity, weight gain is associated with increased lipogenesis, resulting in large part from stimulated expression of lipogenic enzymes, such as fatty acid synthase (FAS) (Diraison et al. 2002). As such, promotion of fatty acid uptake and oxidation, along with decreasing endogenous fatty acid synthesis, have become attractive potential anti-obesity strategies (Niu et al. 2012).

Investigation into the efficacy and mechanism of action of herbal medicines on body weight and composition has been gaining considerable attention (Lenon et al. 2012; Sui et al. 2012). For example, the medicinal anti-diarrheic drug *Geranium thunbergii* has been reported to improve high-fat diet-induced obesity by altering adipokine levels and

down-regulating the expression of lipogenic enzymes (Sung et al. 2011). Berberine has been shown to reduce fat mass by promoting fat oxidation (Lee et al. 2006). Recent studies on LWDH (Xue et al. 2006; Qian et al. 2010; Perry et al. 2012) and its ethanol extract (unpublished data from the Wang lab) have demonstrated its efficacy on lowering body weight and improving body fat composition, as well as lipid, FFA, and various adipokine levels. To date, however, no investigation into the potential mechanism of action responsible for these effects had been conducted.

Activation of metabolic pathways which promote the transport of fatty acids across the mitochondrial plasma membrane for oxidation have been shown to prevent fatty acid accumulation in various tissues (Kahn et al. 2005). It was once believed that this process occurred by way of passive diffusion (Kiens and Roepstorff 2003). More recent evidence, however, has indicated that this process takes place in a protein-mediated fashion (Bonen et al. 2007). One such protein cascade that regulates lipid homeostasis and fatty acid oxidation is driven by the heterotrimeric enzyme AMP-activated protein kinase (AMPK) (Niu et al. 2012). When activated, the AMPK cascade inhibits anabolic and promotes catabolic pathways of energy metabolism (Carling 2005). Activated (phosphorylated) AMPK phosphorylates (and inactivates) the downstream target, acetyl-CoA carboxylase (ACC), and thus decreases the production of malonyl-CoA (Harwood et al. 2003). Malonyl-CoA is an inhibitor of carnitine palmitoyltransferase-1 (CPT1), the rate-limiting enzyme responsible for the transport of fatty acids into the mitochondrion for beta-oxidation (McGarry 2002). Reduced malonyl-CoA production results in a release of the inhibitory effect on CPT1, promoting the transfer of fatty acids into the mitochondrion for beta-oxidation, as well as in a

concomitant suppression of fatty acid synthesis (Kahn et al. 2005). As such, activation of the AMPK-mediated fatty acid oxidation cascade has been emerging as a promising anti-obesity treatment strategy (Kahn et al. 2005; Niu et al. 2012).

The aim of the present study was to investigate the efficacy of LWDH and its ethanol extract on fatty acid oxidation and synthesis as a potential body weight-lowering mechanism of action of the product. Gene and protein expressions of key targets in the AMPK-mediated fatty acid oxidation cascade, including AMPK, pAMPK, ACC, pACC, and CPT1, and the lipogenic enzyme, FAS, were measured.

5.3. Materials and methods

5.3.1. In vivo study

5.3.1.1. Animals and diet

The animals and experimental diet used in the present study were as described in Chapter 2. Sixty male rats were purchased from Charles River Laboratories (Montréal, QC, Canada). Twelve of these animals were of the obesity-resistant strain (OR-CD) and 48 were of the obesity-prone strain (OP-CD). All animals were housed individually in cages in a temperature-controlled room with a 12 hour light:dark cycle and acclimated for 2 weeks with free access to regular rodent chow and water. The OR-CD rats served as the normal control group (OR) and the OP-CD rats were randomly divided into 4 groups (n = 12 each) prior to the commencement of treatment. Randomization of the OP-CD rats was accomplished by ordering the rats based on initial body weight from lightest to heaviest and assigning rats sequentially to 1 of 4 groups. These groups included an obesity control (OP) and 3 treatments (T1A, T1B, and T1C).

The T1A, T1B, and T1C groups were treated for 9 weeks by twice-daily oral gavage with total daily dosages of 500, 1500, or 3500 mg/kg body weight (BW) of the concentrated LWDH pills (Wanxi Pharmaceuticals Co. Ltd., Henan, China) suspended in water and referred to respectively as T1A, T1B, and T1C. The OR and OP control groups received water via oral gavage as the control vehicle. Treatment administration was performed during the light cycle; once in the morning and again 6 hours later. Although rats are generally more active and thus exert more energy during the dark period (Zucker 1971), unpublished data from the Wang lab shows that LWDH enhances energy expenditure at a larger magnitude during the light period. Throughout the treatment period, all rats were fed an AIN-93G diet (Research Diets Inc., New Brunswick, NJ, USA), modified to contain 60% energy from fat, which was provided in the form of a lard and sunflower oil mixture at a ratio of 96 to 4% (w/w). Body weight and food intake were recorded on a daily basis. Daily food intake was calculated by subtracting the total food plus dish weight from that of the previous day. At the end of the study, all animals were fasted overnight and anaesthetized using Isoflurane (Abraxis BioScience, Richmond Hill, ON, Canada). Blood samples were collected via left ventricular cardiac puncture, placed on ice, and allowed to clot. After centrifugation at 2,500 g at 4ºC for 15 minutes, serum was collected, aliquoted, and stored at -80°C until analysis of each biomarker was conducted. Liver and muscle tissue samples were excised and rinsed briefly in 1 X PBS (Sigma), frozen immediately in liquid nitrogen, and stored at -80°C until analysis. The animal use and experimental protocols were approved by the Joint Animal Care and Research Ethics Committee of the National Research Council Canada and the University of Prince

Edward Island. The study was conducted in accordance with the guidelines of the Canadian Council on Animal Care.

5.3.1.2. Preparation and dose determination of LWDH pill

The LWDH concentrated pills used in the present study were manufactured by Henan Wanxi Pharmaceuticals Ltd. Co. (Nanyang, Henan, China) using the 6 Chinese herbs described in Chapter 2 of this thesis, at a composition of 160 g RRP, 80 g RD, 80 g FC, 60 g CM, 60 g RA, and 60 g Poria. To ensure homogeneity, the pills were produced in accordance with the China Pharmacopoeia standard of quality control (Fu et al. 2009). Briefly, following extraction with 95% ethanol, the residue of CM was mixed with all RRP, RA, and Poria, and part of FC (27 g), followed by extraction with hot water (twice, 2 hours each). The water extract was then concentrated to form a paste, followed by mixing with powdered RD, FC (53 g), and the ethanol extract of CM to form the final product. Based on the recommended daily dosage of 24 pills (approximately 4.5 g/day) in humans and the previously reported dosage of 2.4 g/kg/day in rats (Xue et al. 2005), 3 dosages of 500, 1500, and 3500 mg/kg BW were chosen for the present study. The daily stomach gavage capacity of the rats used in the present study was determined to be approximately 6 mL (University Veterinarian, personal communication). Thus, daily treatments were prepared in a vehicle volume of 6 mL, administered twice-daily (approximately 3 mL per gavage session), and adjusted based on daily body weight recordings.

5.3.1.3. Rat liver and muscle tissue gene expression

In vivo expression of genes controlling fatty acid oxidation and synthesis was conducted by quantitative real-time polymerase chain reaction (qPCR) using liver and muscle tissues of OR and OP control rats and T1C treatment rats.

5.3.1.3.1. Rat liver and muscle tissue RNA isolation

Isolation of RNA from rat liver and muscle tissues was performed by way of homogenization using the Trizol method, as previously reported (Rio et al. 2010). In brief, 1 mL Trizol reagent (Sigma-Aldrich Ltd., Oakville, ON, Canada) was added per 100 mg frozen tissue to a 2 mL round-bottom tube (Sigma) and homogenized thoroughly using a PowerGen 125 homogenizer (Fisher Scientific, Ottawa, ON, Canada). Once homogenized, approximately 200 µL chloroform (Sigma; 1/5 the Trizol reagent volume) was added and the tubes were mixed vigorously using a bench top Mini Vortexer (Analytical Instruments, Golden Valley, MN, USA). Following incubation at room temperature and centrifugation at 10,000 g at 4ºC for 10 minutes using a bench top Mini Spin centrifuge (Eppendorf, Mississauga, ON, Canada), the aqueous phase was transferred to a clean 1.7 mL tube (Sigma). After addition of 500 µL isopropanol (Sigma; 1/2 the Trizol reagent volume), vigorous vortexing, incubation at room temperature, and centrifugation at 12,000 g at 4ºC for 10 minutes, the isopropanol was removed. The remaining RNA pellet was washed with 1 mL 75% EtOH (diluted in diethylpyrocarbonate (DEPC) H_2O; Merck, Darmstadt, Germany), vortexed briefly, and centrifuged at 10,000 g at 4ºC for 10 minutes. After removal of the 75% EtOH, the RNA pellet was allowed to dry at room temperature. The dried pellet was then dissolved in

DEPC H_2O. The samples were stored at -20ºC or processed immediately for the measurement of RNA concentration and purity.

5.3.1.3.2. Measurement of rat liver and muscle tissue RNA concentration and purity

Concentration and purity of RNA isolated from rat liver and muscle tissues were measured using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). An aliquot of 2 µL liver and muscle RNA samples dissolved in DEPC H_2O was used for the measurement of RNA concentration (ug/mL) and purity (A_{260}/A_{280}). The cut-off for RNA purity was 1.8. Blank measurements were performed using 2 µL DEPC H_2O.

5.3.1.3.3. Synthesis of complementary DNA (cDNA)

For the synthesis of cDNA, enough RNA sample to obtain 2 µg cDNA, 4 µL qScript cDNA SuperMix (Quanta BioSciences Inc., Gaithersburg, MD, USA), and enough DEPC H_2O to reach a final volume of 20 µL was added to 200 µL reaction tubes (Sigma), vortexed, and centrifuged briefly. Reverse transcription to cDNA was performed using a thermocycler (Thermo Scientific) and under the following reaction conditions: 5 minutes at 25ºC, 30 minutes at 42ºC, 5 minutes at 85ºC, and a hold period at 4ºC. Following reverse transcription, the samples were diluted using DEPC H_2O. If not used immediately following reverse transcription and dilution, the samples were stored temporarily at -20ºC.

5.3.1.3.4. Quantitative real-time polymerase chain reaction (qPCR)

Quantitative real-time polymerase chain reaction was performed using 3 µL diluted cDNA and 12 µL qPCR MasterMix (7.5 µL SYBR Green SuperMix (Quanta BioSciences), 2.5 µL DEPC H_2O, 1 µL forward primer, and 1 µL reverse primer). The MasterMix was vortexed and centrifuged briefly prior to adding to the diluted cDNA samples. The qPCR reaction was carried out in strip PCR tubes (Roche Applied Science, Indianapolis, IN, USA) using a Corbett Rotor-Gene 6000 real-time rotary analyzer (Corbett Life Science, Concorde, NSW, Australia) and under the following conditions, slightly modified from those previously reported (Reja et al. 2002): initial denature at 94ºC for 180 seconds followed by 40 cycles at 94ºC for 15 seconds, 57ºC for 20 seconds, and 72ºC for 25 seconds. Fold changes in the expression level of each target gene in response to treatment was calculated using the deltadelta CT method, as previously reported (Livak and Schmittgen 2001), and normalized to the internal control (β actin). The primer sequences of the internal control and genes of interest (AMPK, ACC, CPT1, and FAS) were obtained from the literature (Kim et al. 2008), blasted to determine the sequence homology between species, and purchased from Sigma Genosys (Oakville, ON, Canada). The forward and reverse sequences for each primer are presented in APPENDIX C. Raw data are presented in APPENDIX E.

5.3.1.4. Rat liver and muscle tissue protein expression

In vivo expression of proteins controlling fatty acid oxidation and synthesis was conducted by Western blot using liver and muscle tissues of OP control and T1C rats.

5.3.1.4.1. Rat liver and muscle tissue protein extraction

In brief, 1 mL prepared radioimmunoprecipitation assay (RIPA) buffer containing protease inhibitor was added per 100 mg frozen tissue to a 2 mL round-bottom tube (Sigma) and homogenized thoroughly using a PowerGen 125 homogenizer (Fisher Scientific). Once homogenized, the tubes were centrifuged at 13,000 g at 4ºC for 20 minutes. The supernatant containing the soluble protein was then transferred to clean 1.0 mL tubes (Sigma).

5.3.1.4.2. Rat liver and muscle tissue protein concentration measurement

Protein concentration was measured by bicinchoninic acid (BCA) assay, as previously reported (Brooks et al. 1995). In brief, soluble protein samples were added at a ratio of 1:20 to BCA working reagent (Thermo Scientific) in a 96-well plate (BD Biosciences). Standards and blanks prepared from a stock of bovine serum albumin (BSA; Thermo Scientific) and RIPA buffer (Sigma), respectively, were run in parallel with the samples. The plate was covered, shaken for 30 seconds, and incubated at 37ºC for 30 minutes. The plate was then allowed to cool to room temperature and the absorbance was read at 562 nm on a Varioskan Flash multimode reader (Thermo Scientific). Standard curves were generated for the calculation of protein concentration of each sample and are presented in APPENDIX F. All samples were then standardized using prepared RIPA buffer and stored at -20ºC in preparation for Western blot experiments.

5.3.1.4.3. Western blot

Western blotting was performed using standardized protein samples diluted 1:1 with sample buffer (2 X Laemmli buffer; Bio-Rad Laboratories, Mississauga, ON, Canada) consisting of 50 µL β-mercaptoethanol (Sigma) in 950 µL Laemmli buffer. The diluted samples were boiled for 5 minutes using a block heater, cooled on ice, vortexed, and centrifuged at 1,200 g at room temperature for approximately 2 minutes. Prior to beginning the experiments, polyacrylamide gels (resolving and stacking) were prepared using the recipes presented in APPENDIX F. Once polymerized, the gels were placed in an electrode assembly tank, filled with 1 X running buffer (diluted from 5 X running buffer containing 15 g Tris base, 74 g glycine, 50 mL 10% sodium dodecyl sulfate; SDS (all from Sigma), and enough water to reach a final volume of 1 L) and monitored for any leakage. The running buffer was adjusted to pH 8.3 prior to loading. The gels were run at 75 volts (V) for approximately 30 minutes or until the samples had visibly passed through the stacking gel. The voltage was then increased to 150 V and run for an additional 60-90 minutes. For larger proteins, the run time was lengthened and optimized accordingly. Approximately 15-30 minutes prior to completion of the gel electrophoresis, pre-cut pieces of filter paper and nitrocellulose membrane (Life Technologies) were equilibrated by soaking in 1 X transfer buffer (3.03 g Tris base, 15.01 g glycine, 200 mL methanol, and 800 mL water) under conditions of gentle shaking on a plate rocker. The proteins were then transferred from the gel to the nitrocellulose membrane at 15 V for approximately 60 minutes. To detect the protein of interest, membranes were first blocked using 5% milk (powdered) dissolved in tris-buffered saline with Tween (TBST; Sigma) for 60 minutes at room temperature or overnight at 4ºC. The membranes were then rinsed 3 times with TBST for 10 minutes

each on a plate rocker and incubated for approximately 60 minutes at room temperature with primary (1º) antibody (all from Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) diluted in TBST. Following incubation with 1º antibody, the membranes were rinsed 3 times with TBST for 10 minutes each on a plate rocker and incubated for approximately 60 minutes at room temperature with horse radish peroxidase (HRP)-conjugated secondary (2º) antibody (Santa Cruz or Jackson ImmunoResearch Inc., West Grove, PA, USA) diluted in TBST. Dilution factors and host sources for the 1º and 2º antibodies against the proteins of interest are presented in APPENDIX H. Following incubation with 2º antibody, the membranes were rinsed 3 times with TBST for 10 minutes each on a plate rocker, covered with 1.0 mL detection reagent (Thermo Scientific), and incubated for approximately 1 minute at room temperature. The protein bands of interest were then visualized using a ChemiDoc XRS system and quantified using Bio-Rad Quantity One software. All Western blot materials were purchased from Bio-Rad, unless otherwise specified.

5.3.2. In vitro study

5.3.2.1. Preparation and concentration determination of LWDH ethanol extract

The ethanol extract of LWDH used in the present study was prepared from the standardized commercial concentrated LWDH pill described and used *in vivo* throughout this thesis, as previously reported (Sangha et al. 2012): dry unpolished pills were milled and extracted twice with 95% ethanol (30 minutes each). Removal of the solvent under reduced pressure and drying at 70°C yielded the ethanol extract of LWDH used in the present study. Chemical profiling of the prepared ethanol extract of LWDH

using various analytical techniques has recently been published (Sangha et al. 2012). The major compounds elucidated from the extract are presented in APPENDIX D.

Concentration determination of the ethanol extract of LWDH in both HepG2 cells and L6 myotubes was determined by MTT assay (Life Technologies, Inc.). A LWDH ethanol extract stock solution was made by dissolving the dried extract in DMSO (Sigma) and treatment concentrations ranging from 0 to 200 µg/mL were prepared in the cell type-specific complete medium. The concentration of DMSO in each treatment was adjusted to 0.05%. Next, cells were cultured in 96-well plates (BD Biosciences, Mississauga, ON, Canada). The media was aspirated and treated with 200 µL of the prepared LWDH ethanol extract concentrations. After an overnight (24 hours) incubation at 37ºC, 20 µL of the MTT stock solution (5 µg/mL) was added to each well and incubated for 4 hours at 37ºC. Following incubation, the media was aspirated and 200 µL DMSO was added. The plate was then covered and shaken for 5 minutes on a plate rocker (Thermo Scientific) and the absorbance was read at 570 nm on a Varioskan Flash multimode reader (Thermo Scientific).

5.3.2.2. HepG2 cell and L6 myotube gene expression

In vitro expression of genes controlling fatty acid oxidation and synthesis was conducted by qPCR using HepG2 cells and L6 myotubes.

5.3.2.2.1. Culture of HepG2 cells

HepG2 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured at 37ºC with 5% CO_2 in complete growth media consisting of Dulbecco's Modified Eagle Medium (DMEM; Sigma)

supplemented with 10% heat-inactivated FBS (Sigma) and 1% antibiotic (Penicillin/Streptomycin; Sigma). Media was changed every 2-3 days and cells were treated upon reaching a confluency of 70-80%.

5.3.2.2.2. Treatment of HepG2 cells with LWDH ethanol extract

Cells were grown in 75 cm^2 flasks (BD Biosciences). Upon reaching ~80% confluency, the media was removed and the cells were washed with 1 X PBS (Sigma). Trypsin (Cellgro, Manassas, VA, USA) was then added to detach the cells. When the cells were detached, fresh media was added to inactivate the trypsin. The flask contents were then transferred to 50 mL conical tubes (BD Biosciences). Following centrifugation at 10,000 g at 4ºC for 5 minutes, the media was removed and the cells were re-suspended in fresh media. The cells were counted using a hemocytometer (Hausser Scientific, Horsham, PA, USA) and diluted to a density of 4 x 10^5 cells/mL. An aliquot of 2 mL was added to each well to achieve the desired cell density of 8 x 10^5 cells/mL. The media was changed every 2-3 days. Once confluent, the cells were treated overnight (24 hours) at 37ºC with the LWDH ethanol extract at concentrations ranging from 0 to 100 μg/mL.

5.3.2.2.3. Culture of L6 myotubes

Cells were cultured at 37ºC with 5% CO_2 in complete growth media consisting of DMEM (Sigma) supplemented with 10% heat-inactivated FBS (Sigma) and 1% antibiotic (Sigma). Once confluent, the growth media was replaced with differentiation media consisting of DMEM (Sigma) supplemented with 2% horse serum

(Sigma) and 1% antibiotic (Sigma). Media was changed every 2-3 days and cells were treated upon differentiation (7-9 days).

5.3.2.2.4. Treatment of L6 myotubes with LWDH ethanol extract

Cell culture conditions and treatment regimes for L6 myotubes were as for HepG2 cells, with minor modifications: L6 myotubes were seeded (2.5 x 10^5 cells/mL) and allowed to differentiate prior to treatment. To simulate the effect of a high-fat diet *in vitro*, L6 myotubes were pre-treated with, or without, fatty acid (palmitate; 0.25 mM/L; Sigma) in DMEM containing 0.5% (w/v) fatty acid-free BSA (Sigma) prior to treatment with the LWDH ethanol extract at a concentration range of 0 to 200 µg/mL.

5.3.2.2.5. HepG2 cell and L6 myotube RNA isolation

Isolation of RNA from HepG2 cells and L6 myotubes was performed using the Trizol method, as previously reported (Rio et al. 2010). In brief, after aspiration of the media, 1 mL Trizol reagent (Sigma) was added to each well and the plates were kept at room temperature for 10 minutes. The well contents were transferred to 1.7 mL tubes (Sigma), followed by addition of 200 µL chloroform (Sigma). The tubes were vortexed vigorously, incubated at room temperature for 10 minutes, and centrifuged at 12,000 g at 4ºC for 10 minutes. The top aqueous phase was transferred to a new 1.7 mL tube (Sigma) and 500 µL isopropanol (Sigma) was added. The tubes were again vortexed vigorously, kept at room temperature for 10 minutes, and centrifuged at 12,000 g at 4ºC for 10 minutes. Following removal of the isopropanol, the RNA pellet was washed with 1 mL DEPC H_2O-diluted EtOH (75%), vortexed, and centrifuged at 10,000 g at 4ºC for 10 minutes. After removal of the 75% EtOH, the RNA pellet was allowed to dry at room

temperature. The dried pellet was then dissolved in DEPC H_2O. The samples were stored at -20ºC or processed immediately for measurement of concentration and purity.

5.3.2.2.6. Measurement of HepG2 cell and L6 myotube RNA concentration and purity

RNA concentration and purity from HepG2 cells and L6 myotubes was determined as previously described for rat liver and muscle tissues.

5.3.2.2.7. Synthesis of complementary DNA (cDNA)

Synthesis of cDNA from HepG2 cell and L6 myotube RNA was performed as previously described for rat liver and muscle tissues.

5.3.2.2.8. Quantitative real-time polymerase chain reaction (qPCR)

Quantitative real-time polymerase chain reactions using HepG2 cell and L6 myotube cDNA was performed as previously described for rat liver and muscle tissues.

5.3.2.3. HepG2 cell and L6 myotube protein expression

In vitro expression of proteins controlling fatty acid oxidation and synthesis was conducted by Western blot using HepG2 cells and L6 myotubes.

5.3.2.3.1. HepG2 cell and L6 myotube protein extraction

In brief, the media was removed and the plates were washed twice with cold 1 X PBS, followed by addition of 100 µL cold 1 X RIPA buffer per well. The cells were then scraped and the well contents were transferred to clean 1.7 mL tubes and kept

on ice for approximately 5 minutes. The tubes were then sonicated for approximately 2 minutes, followed by centrifugation at 12,000 g at 4ºC for 10 minutes in order to pellet the undesired cellular debris. The supernatant containing the soluble protein was then transferred to new tubes, on ice, in preparation for protein concentration measurement and standardization.

5.3.2.3.2. HepG2 cell and L6 myotube protein concentration measurement

Measurement of protein concentration from HepG2 cells and L6 myotubes was performed as previously described for rat liver and muscle tissues.

5.3.2.3.3. Western blot

Western blot using HepG2 cell and L6 myotube protein samples was as previously described for rat liver and muscle tissues. Dilution factors and host sources for the 1º and 2º antibodies against the proteins of interest are presented in APPENDIX H.

5.3.3. Statistical analyses

Data analyses were performed by one-way ANOVA using SAS 9.2 statistical software (SAS Institute, Cary, NC, USA). Differences between treatment means were determined by pair-wise comparisons using the least squares means test, with $P < 0.05$ indicating statistical significance. Results are presented as mean values with their standard errors.

5.4. Results

5.4.1. In vivo study

5.4.1.1. Gene expression in liver tissue of obese rats

The effect of LWDH on fatty acid oxidation and synthesis target gene expression in liver tissue of obese rats was assessed after the 9 week treatment period and is presented in Figure 5-1. Levels of AMPK mRNA in the liver were significantly reduced ($P = 0.02$) in response to high-fat diet feeding, but the high dose LWDH treatment was unable to reverse this effect ($P = 0.4$). Levels of ACC mRNA in the liver were reduced in response to high-fat diet feeding and reversed towards control levels in response to high dose LWDH treatment. However, these effects were not statistically significant. High-fat diet feeding significantly reduced ($P = 0.04$) gene expression of CPT1 in the liver, which was reversed by high dose LWDH treatment. This effect, however, did not reach statistical significance ($P = 0.09$). Gene expression of FAS in the liver was significantly elevated ($P = 0.03$) in response to high-fat diet feeding and reversed ($P = 0.04$) by high dose LWDH treatment.

5.4.1.2. Gene expression in muscle tissue of obese rats

The effect of LWDH on fatty acid oxidation and synthesis target gene expression in muscle tissue of obese rats is presented in Figure 5-2. Muscle tissue gene expression of these targets was similarly affected as found in the liver of obese rats, differing only in the effect of high dose LWDH treatment on the gene expression of CPT1. Levels of the CPT1 gene were decreased by high-fat diet feeding ($P = 0.03$) and significantly reversed by high dose LWDH treatment ($P = 0.02$).

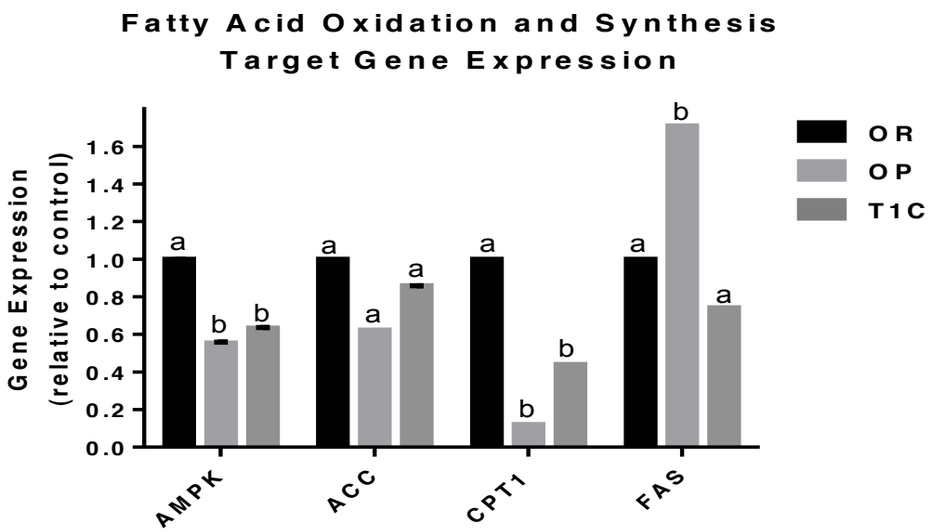


Figure 5-1: Effect of LWDH pill on fatty acid oxidation and synthesis target gene expression in liver tissue of obese rats. OR: normal control; OP: positive control; T1C: 3500 mg/kg/day LWDH-treated OP. All animals were fed a high-fat diet AIN-93G diet modified to contain 60% energy from fat). Data were analyzed by one-way ANOVA. Differences between treatment means were determined by pair-wise comparisons using the least squares means test, with P <0.05 indicating statistical significance. Data are expressed as mean values with their standard errors (n = 6). Means with different superscripts were significantly different.

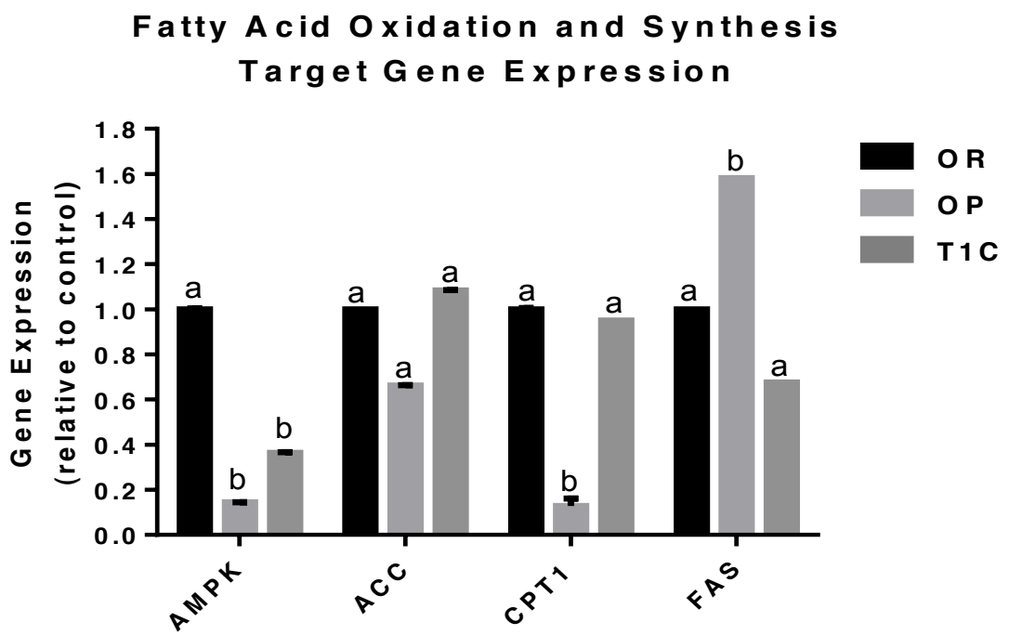
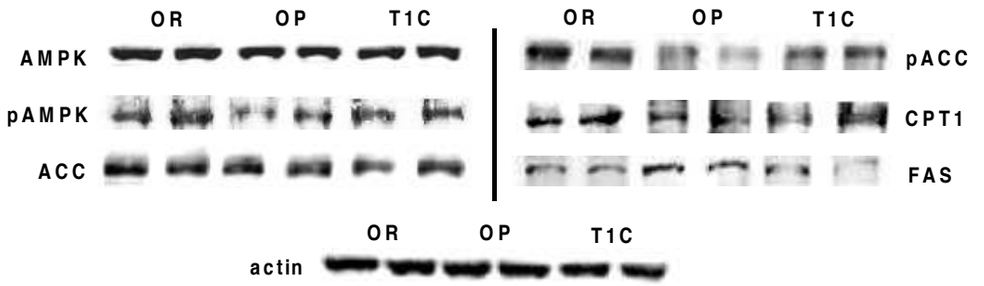


Figure 5-2: Effect of LWDH pill on fatty acid oxidation and synthesis target gene expression in muscle tissue of obese rats. OR: normal control; OP: positive control; T1C: 3500 mg/kg/day LWDH-treated OP. All animals were fed a high-fat diet (AIN- 93G diet modified to contain 60% energy from fat). Data were analyzed by one-way ANOVA. Differences between treatment means were determined by pair-wise comparisons using the least squares means test, with $P < 0.05$ indicating statistical significance. Data are expressed as mean values with their standard errors ($n = 6$). Means with different superscripts were significantly different.

5.4.1.3. Protein expression in liver tissue of obese rats

The effect of LWDH on fatty acid oxidation and synthesis target protein expression in liver tissue of obese rats was assessed after the 9 week treatment period and is presented in Figure 5-3. The protein expression level of AMPK was unaffected by either high-fat diet feeding or high dose LWDH treatment. In contrast, the protein expression level of pAMPK was significantly reduced ($P = 0.001$) in response to high-fat diet feeding but reversed ($P = 0.007$) by high dose LWDH treatment. Similar to AMPK, protein levels of ACC were unaffected by either high-fat diet feeding or high dose LWDH treatment. The protein level of pACC was reduced ($P = 0.01$) in response to the high-fat diet. Reversal of this effect in response to high dose LWDH treatment, however, did not reach statistical significance ($P = 0.08$). The high-fat diet reduced ($P = 0.02$) the liver protein expression of CPT1 but this effect was not significantly reversed by the high dose LWDH treatment ($P = 0.08$). Protein expression of FAS in the liver was increased ($P = 0.04$) by the high-fat diet and marginally reduced ($P = 0.08$) by the high dose LWDH treatment.



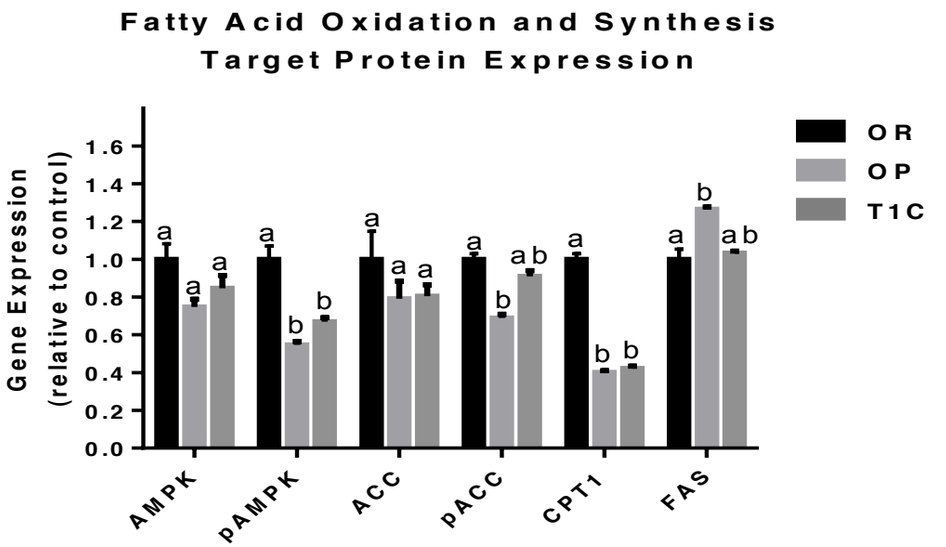


Figure 5-3: Effect of LWDH pill on fatty acid oxidation and synthesis target protein expression in liver tissue of obese rats. OR: normal control; OP: positive control; T1C: 3500 mg/kg/day LWDH-treated OP. All animals were fed a high-fat diet (AIN-93G diet modified to contain 60% energy from fat). Data were analyzed by one-way ANOVA. Differences between treatment means were determined by pair-wise comparisons using the least squares means test, with $P < 0.05$ indicating statistical significance. Data are expressed as mean values with their standard errors ($n = 6$). Means with different superscripts were significantly different.

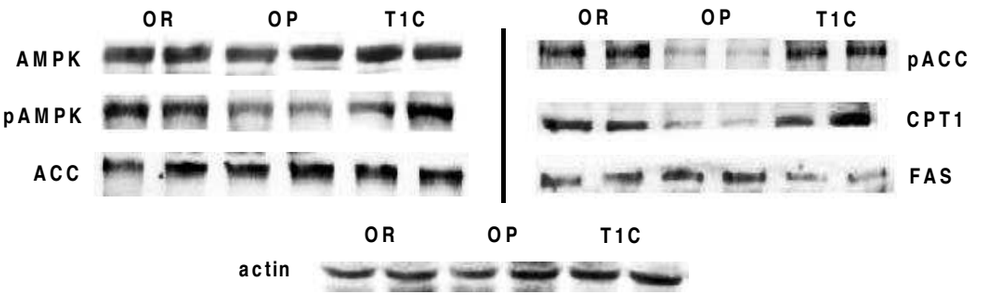
5.4.1.4. Protein expression in muscle tissue of obese rats

The effect of LWDH on fatty acid oxidation and synthesis target protein expression in muscle tissue of obese rats is presented in Figure 5-4. More significant treatment effects were seen in muscle than in the liver tissue. In contrast to the protein expression levels observed in the liver, a significant down regulation of pAMPK ($P = 0.01$), pACC ($P = 0.007$), and CPT1 ($P = 0.004$), but up regulation of FAS ($P = 0.001$) protein expression, was observed in response to high-fat diet feeding. Strikingly, all of these effects were significantly reversed in response to high dose LWDH treatment (pAMPK, $P < 0.001$; pACC, $P = 0.006$; CPT, $P < 0.001$; and FAS, $P < 0.001$).

5.4.2. In vitro study

5.4.2.1. Non-toxic concentration determination of LWDH ethanol extract in HepG2 cells and L6 myotubes

Concentration determination for the ethanol extract of LWDH in both HepG2 cells and L6 myotubes was determined by MTT assay and is presented in Figure 5-5. In HepG2 cells, cell viability remained stable at the concentration range of 0-100 μg/mL, but dropped to approximately 80% when the concentration was increased to 200 μg/mL. As such, concentrations of 0, 25, 50, and 100 μg/mL were chosen for subsequent experiments using HepG2 cells. In L6 myotubes, cell viability was not affected at the concentrations up to 200 μg/mL. Therefore, concentrations of 0, 50, 100, and 200 μg/mL were chosen for subsequent experiments using L6 myotubes.



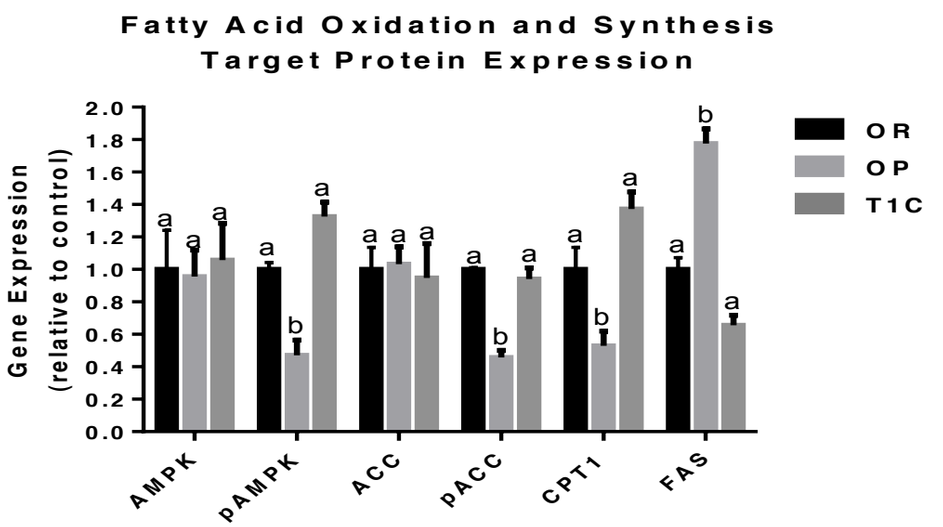


Figure 5-4: Effect of LWDH pill on fatty acid oxidation and synthesis target protein expression in muscle tissue of obese rats. OR: normal control; OP: positive control; T1C: 3500 mg/kg/day LWDH-treated OP. All animals were fed a high-fat diet (AIN-93G diet modified to contain 60% energy from fat). Data were analyzed by one-way ANOVA. Differences between treatment means were determined by pair-wise comparisons using the least squares means test, with $P < 0.05$ indicating statistical significance. Data are expressed as mean values with their standard errors ($n = 6$). Means with different superscripts were significantly different.

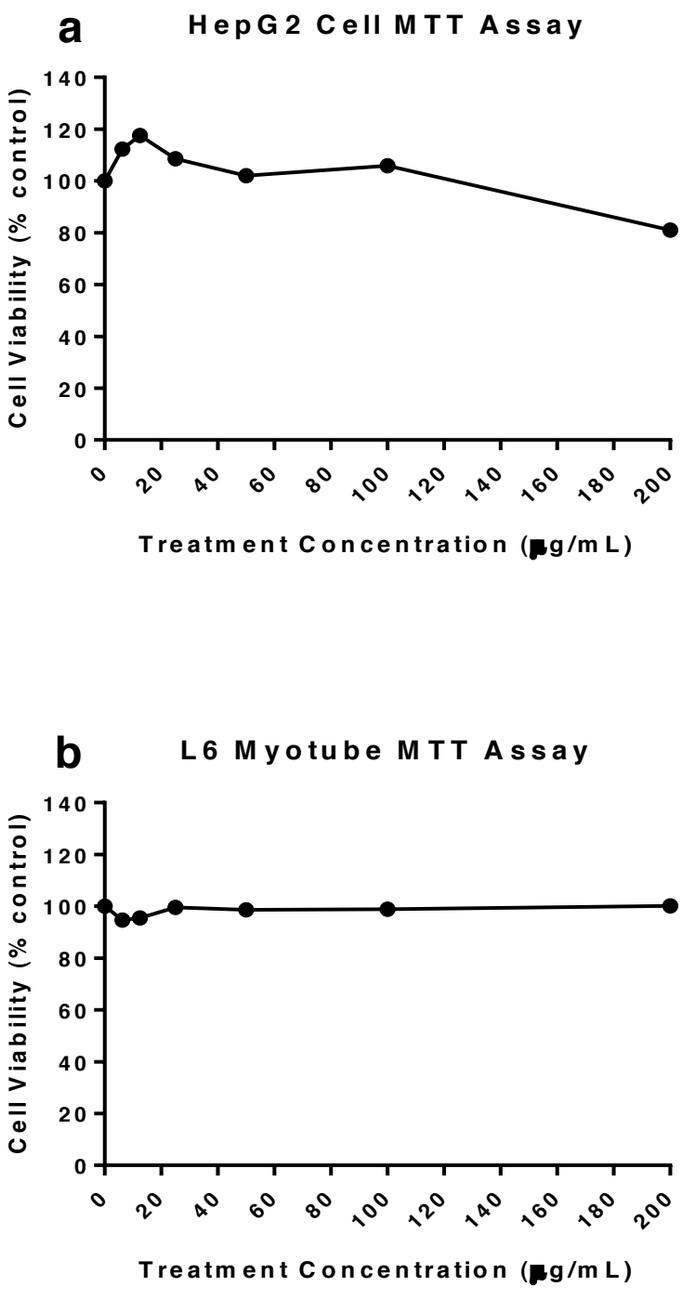


Figure 5-5: Non-toxic concentration determination of LWDH ethanol extract by MTT assay in (a) HepG2 cells and (b) L6 myotubes.

5.4.2.2. Gene expression in HepG2 cells

The effect of the ethanol extract of LWDH on fatty acid oxidation and synthesis target gene expression in HepG2 cells is presented in Figure 5-6. Compared to the control (0 μg/mL), the LWDH ethanol extract did not affect the gene expression levels of AMPK, ACC, CPT1, or FAS.

5.4.2.3. Gene expression in L6 myotubes

The effect of the ethanol extract of LWDH on fatty acid oxidation and synthesis target gene expression in L6 myotubes is presented in Figure 5-7. The control cells were treated with fatty acid (0.25 mM/L palmitate) to simulate the condition in response to high-fat diet feeding in the muscle tissue of obese subjects. Under this experimental condition, treating L6 myotubes with 100 μg/mL of the LWDH ethanol extract led to a significant increase ($P = 0.01$) in AMPK gene expression. Treatment with the 50 and 200 μg/mL LWDH ethanol extract concentrations decreased FAS gene expression ($P = 0.02$ and 0.01, respectively). No treatment effects were observed in ACC or CPT1 gene expressions.

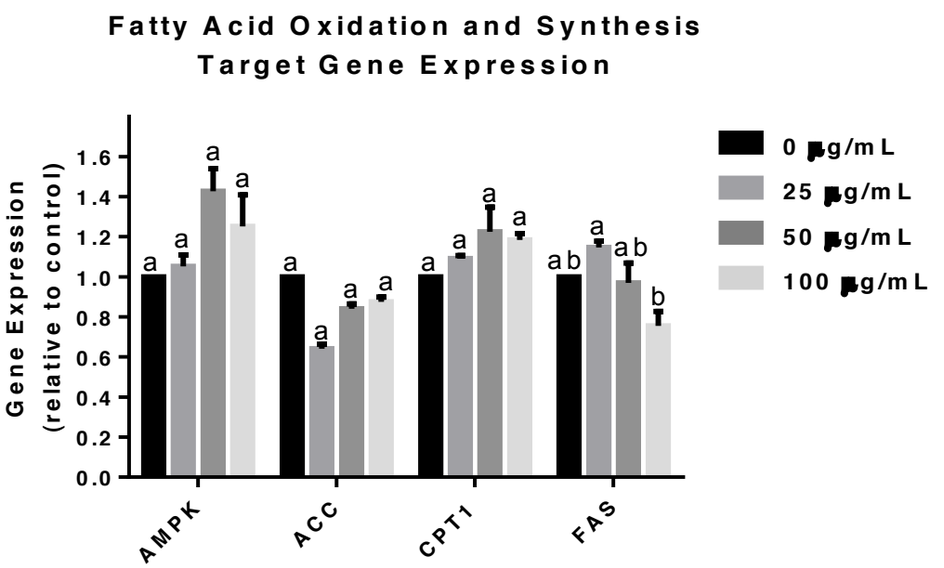


Figure 5-6: Effect of the ethanol extract of LWDH on fatty acid oxidation and synthesis target gene expression in HepG2 cells. Data were analyzed by one- way ANOVA. Differences between treatment means were determined by pair-wise comparisons using the least squares means test, with $P < 0.05$ indicating statistical significance. Data are expressed as mean values with their standard errors ($n = 3$ independent experiments). Means with different superscripts were significantly different.

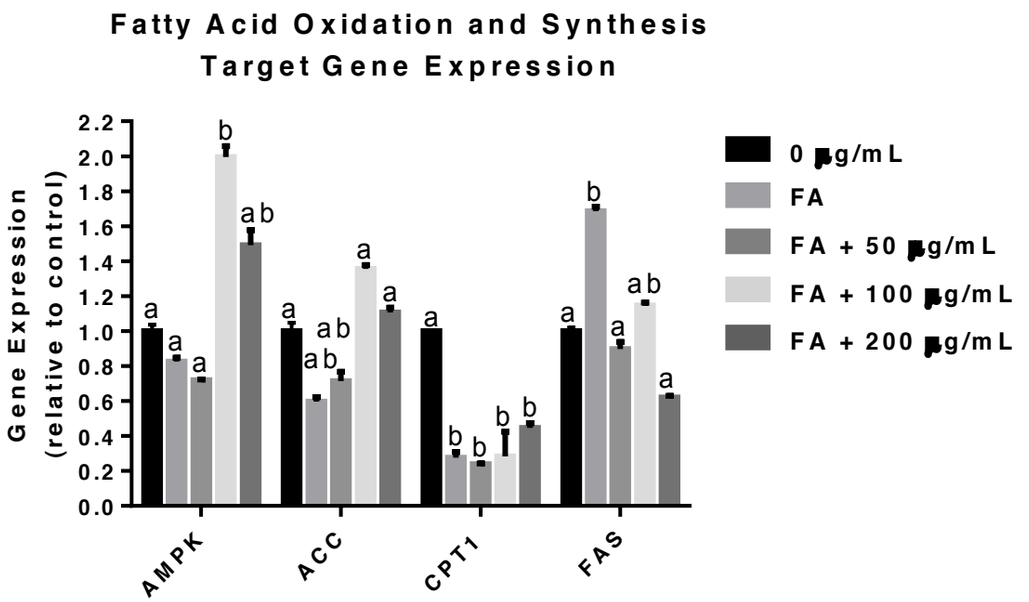


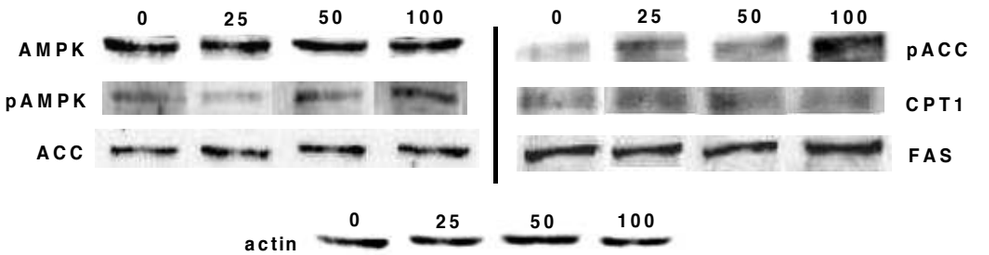
Figure 5-7: Effect of the ethanol extract of LWDH on fatty acid oxidation and synthesis target gene expression in L6 myotubes. FA: fatty acid (palmitate; 0.25 mM/L). Data were analyzed by one-way ANOVA. Differences between treatment means were determined by pair-wise comparisons using the least squares means test, with $P < 0.05$ indicating statistical significance. Data are expressed as mean values with their standard errors (n = 3 independent experiments). Means with different superscripts were significantly different.

5.4.2.4. Protein expression in HepG2 cells

The effect of the ethanol extract of LWDH on fatty acid oxidation and synthesis target protein expression in HepG2 cells is presented in Figure 5-8. Compared to control (0 μg/mL), treatment with the extract did not significantly affect the protein levels of AMPK, ACC, CPT1, or FAS. Treatment with the 50 and 100 μg/mL extract concentrations resulted in significant increases in the protein expressions of pAMPK ($P = 0.01$ and 0.001, respectively) and pACC ($P = 0.01$ and 0.007, respectively).

5.4.2.5. Protein expression in L6 myotubes

The effect of the ethanol extract of LWDH on fatty acid oxidation and synthesis target protein expression levels in FA-treated L6 myotubes is presented in Figure 5-9. Compared to control (0.25 mM/L palmitate), treatment with the ethanol extract of LWDH led to a significant increase in the protein levels of pAMPK (100 μg/mL; $P = 0.03$), pACC (200 μg/mL; $P = 0.01$), and CPT1 (100 μg/mL and 200 μg/mL; $P = 0.01$ and < 0.001, respectively), and a significant decrease in the protein level of FAS (100 μg/mL; $P = 0.004$).



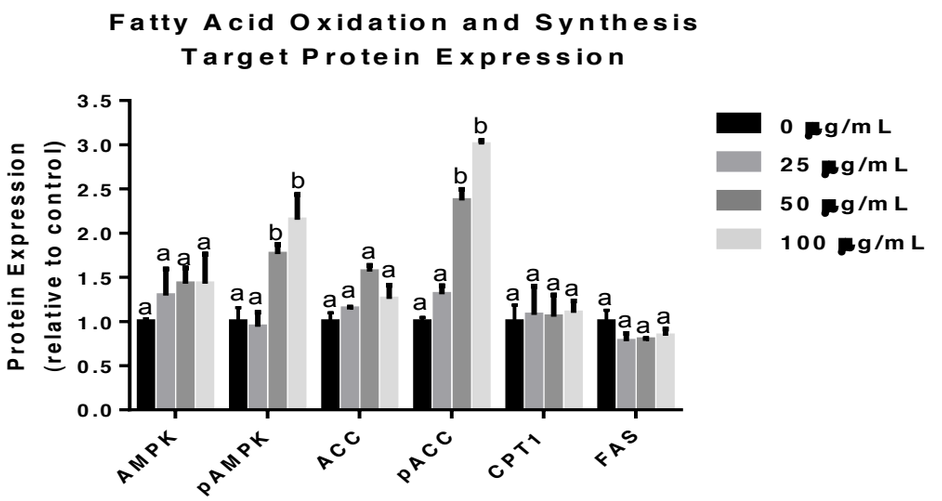


Figure 5-8: Effect of the ethanol extract of LWDH on fatty acid oxidation and synthesis target protein expression in HepG2 cells. Data were analyzed by one-way ANOVA. Differences between treatment means were determined by pair-wise comparisons using the least squares means test, with $P < 0.05$ indicating statistical significance. Data are expressed as mean values with their standard errors ($n = 3$ independent experiments). Means with different superscripts were significantly different.

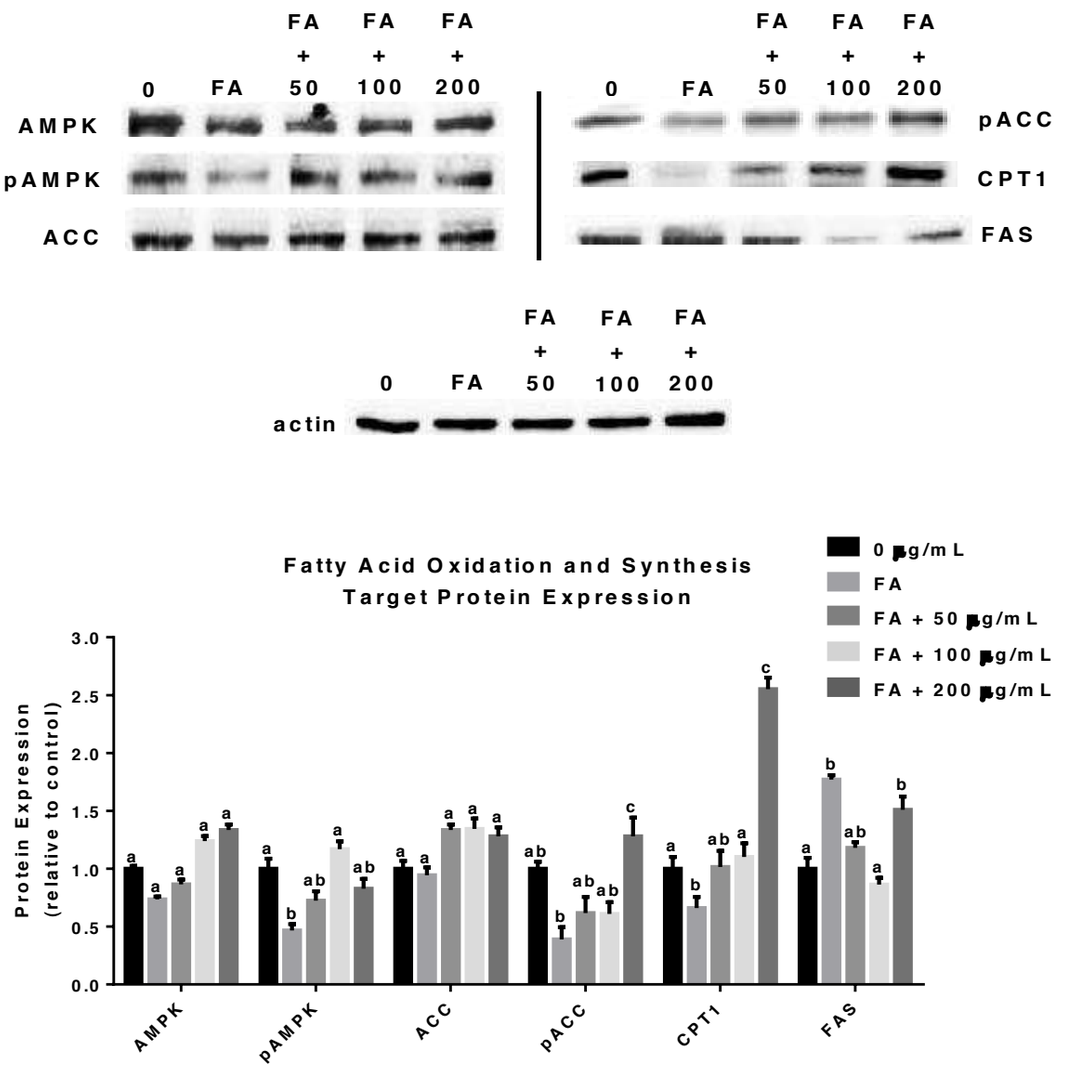


Figure 5-9: Effect of the ethanol extract of LWDH on fatty acid oxidation and synthesis target protein expression in L6 myotubes. FA: fatty acid (palmitate; 0.25 mM/L). Data were analyzed by one-way ANOVA. Differences between treatment means were determined by pair-wise comparisons using the least squares means test, with $P < 0.05$ indicating statistical significance. Data are expressed as mean values with their standard errors (n = 3 independent experiments). Means with different superscripts were significantly different.

5.5. Discussion and conclusions

In obesity, the ability to adjust fat oxidation in response to excessive fat intake is impaired, endogenous fatty acid oxidation capacity is reduced, and induction of genes associated with oxidative capacity is stunted (Ceddia 2005; Boyle et al. 2011). Collectively, these obesity-associated abnormalities in fatty acid oxidation and synthesis have been reported to lead to an increase in circulating and accumulated fatty acids, a myriad of chronic diseases, a decrease in the general quality of life, and a reduction in average life expectancy (Fine et al. 1999; Hu et al. 2001; Mokdad et al. 2003). Fatty acid oxidation and synthesis play an important role in regulating energy metabolism and homeostasis. Consequently, these mechanisms determine circulating TG and FFA levels, fat storage, and ultimately, body weight. Our previous work (Chapter 2) demonstrated that LWDH lowered both TG and FFA levels and prevented weight gain in obese rats (Perry et al. 2012). The results of the present studies are consistent with the notion that LWDH reduces body weight and blood TG levels through increased fatty acid oxidation/decreased fatty acid synthesis.

Activation of metabolic pathways which promote fatty acid oxidation has been shown to prevent fatty acid accumulation in various tissues (Kahn et al. 2005). One pathway that has been garnering significant attention as a mediator of this effect is driven by the master cellular energy sensor AMPK (Ceddia 2005). As an upstream regulator of ACC and CPT1, AMPK is critically involved in fatty acid oxidation and synthesis (Hardie 2004). When AMPK is activated through phosphorylation, it phosphorylates and inactivates the downstream target ACC, a key enzyme responsible for the conversion of acetyl-CoA to malonyl-CoA (Harwood et al. 2003). Malonyl-CoA is an inhibitor of

CPT1, the rate-limiting enzyme responsible for the transport of fatty acids into the mitochondrion for beta-oxidation (McGarry 2002). Malonyl-CoA thus acts as a "switch" between fatty acid synthesis and oxidation. The reduction of malonyl-CoA results not only in a release of the inhibitory effect on CPT1, promoting the transfer of fatty acids into the mitochondrion for beta-oxidation, but also in a concomitant suppression of fatty acid synthesis (Kahn et al. 2005).

Further to the documented alterations in fatty acid oxidation in obesity, weight gain has been shown to be associated with increased lipogenesis, resulting in large part from stimulated expression of the lipogenic enzyme FAS (Diraison et al. 2002). Indeed, FAS is well recognized as a key lipogenic enzyme, rendering it an additionally important therapeutic target for the treatment of obesity (Lin et al. 2007). In addition to promoting fatty acid oxidation, activation of AMPK has been shown to concomitantly inhibit fatty acid synthesis, in large part by way of decreasing FAS expression (Guo et al. 2012). With the known relationship between obesity and impaired fatty acid oxidation/synthesis, beneficial effects on the AMPK-mediated fatty acid oxidation cascade and/or the lipogenic enzyme FAS, either independently or though AMPK activation, have emerged as promising targets for the treatment of obesity (Lin et al. 2007; Niu et al. 2012).

In the present study, LWDH and its ethanol extract were tested *in vivo* and *in vitro*, respectively, for their effects on the AMPK-mediated fatty acid oxidation cascade, as well as the lipogenic enzyme FAS. In liver tissue, the high-fat diet-mediated down regulation of ACC and CPT1 gene expression were reversed toward control levels in response to high dose LWDH treatment. The up regulation in FAS gene expression was significantly reversed back to control levels in response to the same treatment. All other

targets were unaffected in response to either high-fat diet feeding or high dose LWDH treatment. Similar results were seen in gene expression analyses using muscle tissue, but reversal of the high-fat diet-mediated down regulation of CPT1 gene expression in response to high dose LWDH treatment was statistically significant. Western blot analyses *in vivo* were used to assess the effect of LWDH on pAMPK and pACC protein expression. An attenuation in the phosphorylation of AMPK and ACC has been linked to obesity and several of its associated co-morbidities (Tanaka et al. 2005). In its inactive form, ACC, through disinhibition of CPT1, has been shown to promote increased mitochondrial entry of fatty acids and a resultant increase in fatty acid oxidation potential (McGarry 2002). In the liver tissue, pACC and FAS protein expressions were reversed toward control levels in response to high dose LWDH treatment, but these effects were not statistically significant. In the muscle tissue, protein expression levels of pAMPK, pACC, CPT1, and FAS were all significantly reversed back to control levels by high dose LWDH treatment. All other targets were unaffected in response either high-fat diet feeding or high dose LWDH treatment.

Liver and muscle tissue are both recognized as major regulatory sites of whole body fatty acid metabolism (Gallagher et al. 1998). Relevant to the differences in treatment effects seen in muscle compared to liver tissue in the present study, it has been suggested that tissue-directed activation of lipid metabolism is useful for improving and/or managing obesity and several of its related complications (Haramizu et al. 2009). In mice with increased body weight as a result of high-fat diet feeding, markers of fatty acid oxidation have been shown to be more significantly affected in muscle tissue compared to in the liver, with a corresponding increase in susceptibility to the

development of obesity (Haramizu et al. 2009). Furthermore, fatty acid metabolism in muscle tissue has been shown to be more sensitive to increased physical activity and weight loss, during which stored fatty acid utilization increases substantially (Frayn et al. 2006). As such, muscle tissue appears to represent a superior *in vivo* model to liver tissue for investigating treatment modulating effects on key targets of fatty acid oxidation/synthesis.

For investigating the efficacy of natural product extracts on fatty acid oxidation through the AMPK-mediated cascade, HepG2 cells have been used as a representative *in vitro* model of fatty acid metabolism in the liver (Lin et al. 2007; Niu et al. 2012). To simulate the condition of obesity-associated increases in circulating levels of FFA *in vitro*, cells are often pre-treated with varying concentrations of long-chain fatty acids (FA) (Watt et al. 2006). In the present study, non-FA-treated HepG2 cells were used to test the initial efficacy of the ethanol extract of LWDH on key targets of the AMPK-mediated fatty acid oxidation cascade, as well as the lipogenic enzyme FAS. If significant treatment effects were to have been elucidated, transition to a FA pre-treatment design would have been adopted. At the gene level, however, no statistically significant LWDH ethanol extract treatment effects were observed. Protein expression experiments in HepG2 cells showed significant increases in pAMPK in response to the 100 µg/mL LWDH ethanol extract concentration and in pACC in response to the 50 and 100 µg/mL concentrations. It was subsequently decided to select a cell line more representative of the muscle and to pre-treat with FA for subsequent gene and protein expression experiments using the ethanol extract of LWDH.

Similar to HepG2 cells, L6 myotubes have been used as a representative *in vitro* model of fatty acid metabolism in the muscle for investigating the efficacy of natural product extracts on fatty acid oxidation through the AMPK cascade (Watt et al. 2006; Kelly et al. 2010). Using FA-treated L6 myotubes in the present study, expression of the AMPK gene was significantly increased in response to the 100 µg/mL LWDH ethanol extract concentration, while expression of the FAS gene was significantly decreased in response to the 50 and 200 µg/mL concentrations. Protein expression of pAMPK and pACC was significantly increased in response to the 100 and 200 µg/mL concentrations, respectively. Furthermore, FAS levels were decreased in response to the 100 µg/mL concentration of the LWDH ethanol extract.

Upstream activators of AMPK commonly used and reported in the literature include, but are not limited to, the pharmacological agent 5-aminoimida-zole-4-carboxamide riboside (AICAR) and the serine/threonine kinase liver kinase B1 (LKB1) (Shaw et al. 2004; King et al. 2006). Future studies investigating the efficacy of LWDH and/or its ethanol extract on the AMPK cascade using a combination of activators/inhibitors of AMPK may help further elucidate the beneficial effects of the product through increased fatty acid oxidation/decreased fatty acid synthesis. In addition, although the beneficial effects of LWDH and its ethanol extract were seen on several components of the AMPK-mediated fatty acid oxidation cascade, as well as the lipogenic enzyme FAS, dose-dependent effects of the extract were variable. Although the ethanol extract of LWDH was optimized *in vitro* to concentration ranges showing no toxic effect, further optimization in future studies may help limit variations and elucidate more significant and consistent treatment effects.

More recently, a significant amount of research has been conducted on several transcription factors thought to be involved in the gene regulation of fatty acid oxidation/synthesis. Sirtuin-1 (SIRT1) is a nutrient-sensing deacetylase whose levels and activity increase in response to calorie restriction, promoting a more efficient use of energy (Gillum et al. 2010). SIRT1 has been reported to increase expression of fatty acid oxidation genes and decrease expression of lipogenic genes (Gillum et al. 2010). Another key regulator of energy homeostasis that has been garnering significant attention is the transcription factor peroxisome proliferator-activated receptor γ co-activator 1α (PCG-1α). By binding to and modulating the activity of various other transcription factors, PCG-1α can modulate the expression of a number of genes involved in fatty acid oxidation and synthesis alike (Canto et al. 2009; Jeninga et al. 2010). Deacetylation is required for the activation of PCG-1α, and SIRT1 is the only protein identified to be capable of this function (Rodgers and Puigserver 2007). By way of its phosphorylating capabilities, AMPK has also been shown to enhance the ability of SIRT1 to deacetylate (and activate) PCG-1α (Canto et al. 2009). AMPK and SIRT1 have been shown to display condordant functions, such as increased fatty acid oxidation, particularly in muscle tissue (Fulco and Sartorelli 2008). In fact, AMPK and SIRT1 are now known to regulate one another and to have several target molecules in common (Ruderman et al. 2010).

In sum, increased fatty acid oxidation/decreased fatty acid synthesis appears to be strongly representative of the body-weight lowering mechanism of action of LWDH and its ethanol extract. These effects were seen more consistently in muscle tissue and rat L6 skeletal muscle myotubes than in liver tissue and human HepG2 hepatocytes.

CHAPTER 6: OVERALL DISCUSSION, TECHNICAL CONSIDERATIONS, AND CONCLUDING STATEMENT

6.1. Discussion

Obesity is now commonly referred to as the epidemic of the 21st century and is well recognized to confer significant risk for the development of a myriad of associated life threatening complications (Greenberg and Obin 2006). Accumulating evidence suggests, however, that even modest reductions in body weight of as little as 5-10% are sufficient to improve, or even abolish, the majority of obesity-associated health risks (Renehan et al. 2008). In line with the increasing demand to develop safe and efficacious anti-obesity products, there has been a global trend towards the discovery and development of anti-obesity products from natural sources. As a viable complementary/alternative anti-obesity treatment approach, TCM has been garnering significant attention as it is considered to have enormous potential as an information source and starting point for the development of products to reduce obesity (Yin et al. 2008; Perry et al. 2012). The ancient TCM, LWDH, has been used extensively worldwide for general health promotion, but to date, very little investigation into its potential anti-obesity efficacy had been documented.

The objective of Chapter 2 of this thesis was to determine the efficacy of LWDH on body weight and composition, food intake, blood lipids, and leptin and insulin resistance in obese prone rats. Results from this chapter shed light on the potential for the development of LWDH as a natural product for the management of obesity. Treatment with LWDH led to significant reductions in body weight, which persisted throughout the

last 4 weeks of the study, reaching reductions of greater than 8% by the last 2 weeks. In addition, significant reductions in visceral fat deposition and circulating blood lipids, and improvements in leptin and insulin sensitivity were also elucidated. These parameters are all highly related to obesity and an improvement in any one is, in turn, beneficial to weight control and the mitigation of obesity-related complications (Yuan et al. 2007). Further to the beneficial effects on body weight and several associated parameters, LWDH led to significant reductions in food intake. Dietary restriction without malnutrition ameliorates a wide array of age-related complications and prolongs life in a variety of species, including rats (Colman et al. 2009; Ellers et al. 2011). In the present thesis, the possible confounding effect of caloric dilution by LWDH on several investigated parameters, including body weight, was raised. However, it is not believed that caloric dilution influenced the outcomes presented in this thesis and/or in the data published or under consideration for publication in the peer-reviewed literature. Although presented as a weekly average in this thesis, food intake was recorded on a daily basis throughout the *in vivo* study. Food intake during the 2 week acclimation period was consistent and did not differ between control and treatment animals during the entire first week of LWDH treatment by oral gavage. If caloric dilution had influenced body weight or any of the post-sacrifice parameters that were subsequently measured, food intake would have been reduced during the first week of treatment. Furthermore, the high-fat diet that was fed to all experimental animals was administered *ad libitum* throughout the study period, and not restricted to immediately post-gavage. All animals were monitored on a daily basis for general well being throughout the study (with the help of Dr. Jonathon Spears, University Veterinarian, personal communication), and the rate of

stomach emptying did not appear to differ between the control and treatment animals. Furthermore, post-sacrifice liver function testing revealed no adverse side effects in response to LWDH treatment by twice-daily oral gavage. In fact, high-dose LWDH treatment was revealed to have improved liver function. Thus, rather than as a result of caloric dilution, the LWDH-mediated reductions in body weight were believed to be due to one of two potential mechanisms of action, which were investigated in subsequent chapters of this thesis. Chapter 4 investigated decreased energy consumption through appetite regulation as a potential body weight-lowering mechanism of action, while chapter 5 investigated the effects of LWDH and its ethanol extract on key targets of fatty acid oxidation/synthesis.

Prior to investigating the two proposed body weight-lowering mechanisms of action of LWDH and its ethanol extract, chapter 3 of this thesis was conducted to evaluate the efficacy of LWDH on obesity-associated biomarkers of inflammation, oxidative stress, and adiponectin production in obese-prone rats. Obesity is now recognized to be strongly associated with chronic inflammation (O'Rourke 2009), including elevations in the pro-inflammatory cytokine TNF-α, the chronic inflammatory biomarkers CRP, and the acute phase pro-inflammatory cytokine IL-6 (Hotamisligil et al. 1993; Browning et al. 2008). Adipose tissue of obese individuals is also a major source of circulating ROS and has suppressed antioxidant enzyme activity (Furukawa et al. 2004). Furthermore, obesity is associated with dysregulated production of various adipokines, including adiponectin (Yamauchi et al. 2001). Overall, this study demonstrated, for the

first time, the anti-inflammatory, anti-oxidative stress, and adiponectin-ameliorating efficacy of LWDH in OP-CD rats.

The objective of Chapter 4 of this thesis was to determine the efficacy of LWDH and its ethanol extract on appetite regulation by analyzing treatment effects on gut hormones and neuropeptides along the gut-brain axis. Both *in vivo* and *in vitro* experimental designs were employed to test the efficacy of LWDH and its ethanol extract, respectively. Recently, there has been increasingly convincing evidence that weight loss may be due to alterations in the circulating levels and physiology of various gut hormones and neuropeptides (Murphy and Bloom 2004; Cummings and Overduin 2007). Gut hormones act specifically on systems responsible for appetite control, and due to their inherently natural physiological regulation of appetite (Murphy et al. 2006), gut hormone-based therapies are reportedly less likely to cause adverse side effects compared to some of the currently approved drugs (Chaudhri et al. 2008). Overall, under the experimental conditions described in Chapter 4, LWDH and its ethanol extract did not significantly affect key components of the gut-brain axis. Although this was the first study to investigate the efficacy of LWDH on appetite regulation through the gut-brain axis as a potential body weight-lowering mechanism of action, several points have potential to be addressed in future studies. The orexigenic gut hormone, ghrelin, was a key target of investigation in Chapter 4 of this thesis. Ghrelin gene expression was analyzed in ileal tissue of obese rats. However, ghrelin is predominantly expressed and secreted from the stomach, and to a much lesser degree from the small intestine (Date et al. 2000). Unfortunately, stomach tissue was not collected at the time of sacrifice for

subsequent biochemical or molecular analyses. Although the intestinal CaCo-2 cell line has previously been used as a relevant model for studying ghrelin gene expression (Yeung et al. 2006), cultured tissue from the stomach may represent a more physiologically relevant site for the study of ghrelin secretion (Zhao et al. 2010). As such, the CaCo-2 cell line may not have been sufficiently representative of the actual effect of the extract on ghrelin gene expression. In the CNS, the hypothalamic Arc is the key region involved in the regulation of appetite (Murphy and Bloom 2004). Furthermore, expression of NPY, the most potent activator of appetite in the CNS (Valassi et al. 2008), predominates in the Arc region (Williams et al. 2001). At the time of sacrifice, the entire hypothalamic region of the brain was collected and subsequently processed for gene expression analyses. As such, other hypothalamic regions expressing NPY, albeit to a lesser degree, may have confounded the specificity of the results. In addition, although the SH-SY5Y cell line employed for investigating the efficacy of the LWDH ethanol extract on NPY gene expression is representative of the CNS, primary cell cultures using the specific Arc region of the hypothalamus may prove useful for more specific elucidation of the true effects in future investigations. Continued research into the potential to pharmacologically exploit endogenously occurring appetite-modulating gut hormones in an effort to regulate energy homeostasis is required. Nonetheless, there is strong evidence in support of further investigation into the release and function of gut hormones in association with neuropeptides and receptors in the CNS for the development of weight loss or weight maintenance products.

The objective of Chapter 5 of this thesis was to determine the efficacy of LWDH and its ethanol extract on key targets of fatty acid oxidation and synthesis. In obesity, the ability to adjust fat oxidation in response to excessive dietary fat intake is impaired, endogenous fatty acid oxidation capacity is reduced, and induction of genes associated with oxidative capacity is stunted (Boyle et al. 2011). Further to the documented alterations in fatty acid oxidation in obesity, weight gain is often associated with increased lipogenesis (Lewandowski et al. 1998; Strable and Ntambi 2010), resulting in large part from stimulated expression of the lipogenic enzyme FAS (Diraison et al. 2002). Several other factors involved in the regulation of lipogenesis have been identified (Kersten 2001), including nutritional (glycolytic conversion of glucose to acetyl-CoA), hormonal (primarily insulin), and transcriptional (primarily sterol regulatory element binding protein-1c). Collectively, these obesity-associated abnormalities in fatty acid oxidation and synthesis have been shown to lead to increased circulating and accumulated fatty acids and a myriad of obesity-associated co-morbidities (Mokdad et al. 2003). Improvements in leptin and/or insulin sensitivity have been shown to stimulate fatty acid oxidation, correspondingly resulting in a reduction in circulating TG and FFA levels (Gil-Campos et al. 2004). In addition, activation of metabolic pathways which promote fatty acid oxidation has been shown to prevent fatty acid accumulation in various tissues. One such pathway which has been garnering significant attention as a mediator of this effect is driven by the master cellular energy sensor AMPK (Ceddia 2005). With the known relationship between obesity and impaired fatty acid oxidation/synthesis, beneficial effects on the AMPK-mediated fatty acid oxidation cascade and/or the lipogenic enzyme FAS, either independently or though AMPK

activation, represent promising anti-obesity targets (Niu et al. 2012). Overall, increased fatty acid oxidation/decreased fatty acid synthesis appeared to have strong potential as a body-weight lowering mechanism of action of LWDH and its ethanol extract, especially in muscle tissue and FA-treated L6 skeletal muscle myotubes. Nonetheless, several points have potential to be addressed in future studies investigating the body weight-lowering mechanism of action of LWDH and/or its ethanol extract through regulation of key fatty acid oxidation/synthesis targets. Liver and muscle tissue both represent major regulatory sites of whole body fatty acid metabolism (Gallagher et al. 1998). It has also been suggested, however, that tissue-directed activation of fatty acid metabolism is useful for improving and/or managing obesity (Haramizu et al. 2009). Fatty acid oxidation is more significantly affected in muscle compared to in liver tissue (Haramizu et al. 2009). Furthermore, fatty acid metabolism in muscle tissue has been shown to be more sensitive to physical activity and weight loss (Frayn et al. 2006). As a result, muscle tissue and representative muscle cell lines may prove to be more useful models for investigating treatment efficacy on key targets of fatty acid oxidation/synthesis in future studies. Future studies investigating the efficacy of LWDH and/or its ethanol extract on the AMPK cascade using a combination of activators/inhibitors of AMPK may also help to further elucidate the beneficial effects of the product in terms of its potential effects on fatty acid oxidation and synthesis.

6.2. Technical considerations

6.2.1. Dosage optimization

Treatment with the herbal formula LWDH throughout this thesis was by administration of a partially-refined concentrated pill. The high doses chosen for use in this thesis have had precedent in the literature in diabetic rat studies (Xue et al. 2005) and for general health promotion in humans (Hu et al. 2005). The optimal treatment dosages for weight management in rats had, to date, not been investigated and remain to be determined in humans. Nonetheless, the treatment dosages used in this thesis had potential to be significantly reduced when administered in the form of an extract or fraction. As such, an ethanol extract of LWDH was used for further *in vitro* investigation into the potential mechanisms of action of the LWDH-mediated reductions in body weight in Chapters 4 and 5. Although non-toxic treatment concentrations were determined in all of the *in vitro* models used, future studies may benefit from further dosage optimization in an effort to minimize variations and to elucidate more significant and concentration-dependent treatment effects. For instance, as the ethanol extract of LWDH contains considerable amounts of sugar monomers, dimers, and trimers, the concentration could be further reduced by removing the majority of these saccharides. Of the major components elucidated from the LWDH ethanol extract, two in particular may warrant further investigation. Paeonoforin has been reported to have anti-obesity properties (Rabie 2009) and gallic acid has been reported to suppress weight gain by inhibiting pre-adipocyte growth or proliferation (Hsu et al. 2006; Hsu and Yen 2007).

6.2.2. Appetite regulation

As a body weight-lowering mechanism of action of LWDH and/or its ethanol extract, appetite regulation through alterations in key components of the gut-brain axis did not appear to show promising potential under the current experimental conditions. Future investigations into this potential working mechanism would stand to benefit by considering the short half-life of the majority of endogenous gut hormones and selection of the most relevant physiological sites of production/secretion of the targeted parameters. Overall, however, future development of safe and effective anti-obesity treatments through appetite modulation continues to have promising potential.

6.2.3. Regulation of fatty acid metabolism

Increased fatty acid oxidation/decreased fatty acid synthesis appeared to strongly represent a potential body-weight lowering mechanism of action of LWDH and its ethanol extract under the current experimental conditions. As AMPK is the master regulator of this effect, future studies with further targeted activation/inhibition of AMPK may help further elucidate any LWDH- and/or ethanol extract-mediated effects through this mechanism. Investigation into treatment effects on novel transcription factors involved in the regulation of fatty acid oxidation/synthesis may also be warranted.

6.2.4. Choice of reference gene for qPCR experiments

Reference genes typically encode for proteins involved in cell maintenance and are constitutively expressed under most experimental conditions (Mehta et al. 2010). The commonly employed reference genes generally meet this key criterion, but unstable changes in expression may present, depending on the experimental conditions and the

tissue or cell type being analyzed (Lundby et al. 2005). This may affect the normalizing procedure to correct for mRNA content variations between samples (Whelan et al. 2003). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and β-actin have been among the most commonly used reference genes for qPCR experiments (Radonic et al. 2004; Lin and Redies 2012). However, their transcription has been shown to be differentially expressed in various tissues (Jemiolo and Trappe 2004) and it is now known that neither GAPDH nor β-actin mRNA is expressed in all cell or tissue types (Lin and Redies 2012). In the present thesis, β-actin was chosen as the reference gene for all qPCR experiments. Gene expression of β-actin is known to be stably expressed in liver tissue (Sun et al. 2009). In skeletal muscle tissue, however, GAPDH has been reported to be more stably expressed than β-actin (Mahoney et al. 2004). Furthermore, the experimental conditions can affect the expression of different reference genes in the same tissue. In human skeletal muscle following acute exercise, β-actin is more stably expressed that GAPDH following resistance exercise, and vice versa following endurance exercise (Mahoney et al. 2004). Future studies would stand to benefit by normalizing against greater than one reference gene (Bustin et al. 2009), and furthermore, determining and reporting the choice and optimal number of these genes (Vandesompele et al. 2002; Bustin et al. 2009).

6.3. Concluding statement

Control of body weight in an effort to improve or reverse the deleterious obesity-associated effects on the general quality of human life and its enormous economic burden on the health care system is a top priority in nutritional and medical research (Stein and Colditz 2004; Lakdawalla et al. 2005; Perry et al. 2012). This thesis was the first to investigate the efficacy of LWDH against obesity and several of its associated

complications. Our results demonstrated the efficacy and safety of this promising anti-obesity TCM in obese-prone rats. Furthermore, we proposed two potential body weight-lowering mechanisms of LWDH and designed *in vitro* experiments to investigate an ethanol extract of the product. Future studies are required but highly warranted. The technical considerations provided herein may help unlock the full potential of this promising future anti-obesity therapeutic.

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APPENDIX A: COMPOSITION OF MINERAL AND VITAMIN MIXES IN AIN-93G DIET

Table AA-1: Composition of mineral mix in AIN-93G diet (per 1 kg)

Ingredient	Amount (g)
Sodium chloride	259.0
Magnesium oxide	41.9

Magnesium sulfate	257.6
Chromium sulfate	1.925
Cupric carbonate	1.05
Sodium fluoride	0.2
Potassium iodate	0.035
Ferric citrate	21.0
Manganous carbonate	12.25
Ammonium molybdate	0.3
Sodium selenite	0.035
Zinc carbonate	5.6
Sucrose	399.1

Table AA-2: Composition of vitamin mix in AIN-93G diet (per 1 kg)

Ingredient	Amount (g)
Vitamin A palmitate	0.8

Vitamin D_3	1.0
Vitamin E acetate	10.0
Menadione sodium bisulfate	0.08
Biotin	2.0
Cyanococobalamin	1.0
Folic acid	0.2
Nicotinic acid	3.0
Calcium pantothenate	1.6
Pyridoxine-HCl	0.7
Riboflavin	0.6
Thiamin HCl	0.6
Sucrose	978.42

APPENDIX B: FEED EFFICIENCY OF OBESE RATS FED A HIGH-FAT DIET

TABLE AB-1: Feed efficiency of obese rats fed a high-fat diet*

Group	Wk 0	Wk 1	Wk 2	Wk 3	Wk 4	Wk 5	Wk 6	Wk 7	Wk 8	Wk 9

OR	0.40	0.53	0.56	0.72	0.69	1.23	1.01	1.04	2.49	2.19
OP	0.31	0.44	0.42	0.56	0.58	0.81	0.84	0.92	1.80	4.12
T1A	0.38	0.40	0.40	0.61	0.62	0.78	0.80	0.86	1.67	2.33
T1B	0.38	0.39	0.38	0.55	0.54	0.68	0.89	0.83	1.97	2.55
T1C	0.40	0.43	0.41	0.54	0.60	0.77	1.16	1.05	3.53	4.57

*Calculated as a ratio of food intake to weight gain.

APPENDIX C: PRIMER SEQUENCES USED FOR QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION EXPERIMENTS THROUGHOUT THE THESIS

Table AC-1: Primer sequences used for the detection of mRNA expression of appetite regulation genes in rat ileal and hypothalamic tissues

Gene	Forward Primer Sequence	Reverse Primer Sequence

β actin	AGCCATGTACGTAGCCATCC	ACCCTCATAGATGGGCACAG
Ghrelin	CACCAGAAAGCCCAGCAGAGA	CGAAGGGAGCATTGAACCTGA
PYY	GCGGTATGGGAAAAGAGAAGTCC	GCAAGTGAAGTCGGTGTAGTTAGCA
NPY	CCAGACAGAGATATGGCAAG	GTCTTCAAGCCTTGTTCTGG

Table AC-2: Primer sequences used for the detection of mRNA expression of appetite regulation genes in CaCo-2 and SH-SY5Y cells

Gene	Forward Primer Sequence	Reverse Primer Sequence

β actin	AGCCATGTACGTAGCCATCC	ACCCTCATAGATGGGCACAG
Ghrelin	GCAGAGGATGAACTGGAAGTCC	CTCTTCCCAGAGGATGTCCTGA
PYY	CGGACACGCTTCTTTCCAAAACG	TGGTTGGCAGATCTCCCAGGAG
NPY	GCTGCGACACTACATCAACCTC	TGTGCTTTCTCTCATCAAGAGG

Table AC-3: Primer sequences used for measuring mRNA expression of the genes regulating fatty acid oxidation and synthesis in rat liver and muscle tissue

Gene	Forward Primer Sequence	Reverse Primer Sequence

β actin	AGCCATGTACGTAGCCATCC	ACCCTCATAGATGGGCACAG
AMPK	AGGAAGAATCCTGTGACAAGCAC	CCGATCTCTGTGGAGTAGCAGT
ACC	GACGAGCTGATCTCCATCCTCA	ATGGACTCCACCTGGTTATGCC
CPT1	GATCCTGGACAATACCTCGGAG	CTCCACAGCATCAAGAGACTGC
FAS	TTCTACGGATCCACGCTCTTCC	GAAGAGTCTTCGTCAGCCAGGA

Table AC-4: Primer sequences used for the detection of mRNA expression of genes controlling fatty acid oxidation and synthesis in HepG2 cells and L6 myotubes

Gene	Forward Primer Sequence	Reverse Primer Sequence

β actin	AGCCATGTACGTAGCCATCC	ACCCTCATAGATGGGCACAG
AMPK	AGGAAGAATCCTGTGACAAGCAC	CCGATCTCTGTGGAGTAGCAGT
ACC	GACGAGCTGATCTCCATCCTCA	ATGGACTCCACCTGGTTATGCC
CPT1	GATCCTGGACAATACCTCGGAG	CTCCACAGCATCAAGAGACTGC
FAS	TTCTACGGATCCACGCTCTTCC	GAAGAGTCTTCGTCAGCCAGGA

APPENDIX D: MAJOR COMPOUNDS ELUCIDATED IN LWDH ETHANOL EXTRACT

Table AD-1: Major compounds in LWDH ethanol extract separated with C18 column

Ions (*m/z*)	M.W.	Possible Compound

Positive ion	Negative ion		
-	169[M-H]$^-$, 339[2M-H]$^-$	170	gallic acid
127[M+H]$^+$, 109[M+H-H2O]$^+$	-	126	5-hydroxymethyl furfural
359[M+H]$^+$, 197[M+H+H2O-glu]$^+$	403[M+HCOO]$^-$	358	sweroside
229[M+H+H2O-glu]$^+$, 413[M+Na]$^+$	435[M+HCOO]$^-$	390	loganin
498[M+NH4]$^+$	525[M+HCOO]$^-$	480	paeoniforin
167[M+H]$^+$	-	166	paeonol

Table AD-2: Major compounds in LWDH ethanol extract separated with HILIC column

Negative ion (*m/z*)	M.W.	Possible Compound
169[M-H]$^-$,	170,	loganin and gallic acid

435[M+HCOO]$^-$	390 mix	
179[M-H]$^-$, 225[M+HCOO]$^-$, 293[M+Cl]$^-$, 367[M+Cl]$^-$	180, 258, 332 mix	monosaccharide, 5-hydroxymethyl furfual with monosaccharide and gallic acid with monsaccharide
179[M-H]$^-$, 225[M+HCOO]$^-$	180	monosaccharide
295[M-H]$^-$, 455[M-H]$^-$	296, 456 mix	di- and tri-saccharide

APPENDIX E: RAW QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION DATA FROM THE THESIS

Table AE-1: Raw data used for detecting mRNA expression of appetite regulation genes in rat ileal tissue

Gene	OP	T1C

	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3
PYY	20.81	20.73	20.74	22.24	22.48	22.37
	25.51	25.60	25.50	19.30	19.34	19.42
	18.67	18.68	18.79	18.43	18.49	18.51
	19.37	19.46	19.40	21.38	21.57	21.52
	21.81	21.85	22.00	21.74	22.13	21.75
	19.77	18.6	20.15	18.63	18.74	18.72
Ghrelin	35.55	29.90	30.26	27.88	28.46	28.57
	29.31	28.64	29.43	29.40	-	29.67
	28.60	28.01	28.21	26.57	26.70	26.57
	30.07	31.07	30.01	28.09	27.71	27.50
	28.42	29.44	28.63	29.59	29.90	29.52
	25.32	25.35	25.55	29.62	30.34	29.28
β actin	16.48	17.01	17.03	16.65	16.76	16.65
	19.65	19.73	19.85	16.23	16.13	16.13
	16.13	16.12	16.10	15.13	15.03	15.00
	16.13	16.11	16.02	14.21	14.15	14.23
	16.05	15.99	15.98	15.79	15.91	15.78
	14.27	14.29	14.27	15.81	15.99	15.91

Table AE-2: Raw data used for the detection of mRNA expression of appetite regulation genes in rat hypothalamic tissue

Gene	OP			T1C		
	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3
NPY	17.38	17.45	17.42	18.17	18.18	18.18
	18.76	18.64	18.70	17.17	17.27	17.22
	17.32	17.2	17.26	18.88	18.92	18.90
	17.30	17.42	17.36	16.96	17.15	17.06
	17.83	17.91	17.87	17.42	17.49	17.46
	17.92	17.97	17.95	17.42	17.54	17.48
β actin	13.04	13.13	13.09	13.69	13.51	13.60
	13.36	13.36	13.36	13.24	13.28	13.26
	12.89	12.84	12.87	13.46	13.51	13.49
	12.67	12.40	12.54	13.12	12.88	13.00
	13.18	13.16	13.17	13.28	13.35	13.32
	13.19	13.23	13.21	13.34	13.36	13.35

Table AE-3: Raw data used for measuring mRNA expression of appetite regulation genes in CaCo-2 cells by qPCR

Concentration	PYY			Ghrelin			**β actin**		
	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3
0	27.71	29.21	28.46	28.52	28.25	28.385	14.44	14.87	14.66
25	28.79	27.33	28.06	28.31	28.35	28.33	14.84	15.03	14.94
50	27.38	28.23	27.805	28.57	27.97	28.27	15.50	14.85	15.18
100	28.84	27.81	28.33	27.94	28.32	28.13	14.69	14.98	14.84

0, 25, 50, and 100 represent the concentrations of the LWDH ethanol extract in µg/mL.

Table AE-4: Raw data used for measuring mRNA expression of appetite regulation genes in SH-SY5Y cells by qPCR

Concentration	NPY			**β actin**		
	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3
0	19.50	19.78	19.47	12.57	12.70	12.52
5	19.66	19.63	19.45	12.58	12.54	12.56
10	19.76	19.70	19.60	12.77	12.59	12.92
25	19.79	19.79	19.71	12.84	13.36	12.82

0, 5, 10, and 25 represent the concentrations of the LWDH ethanol extract in μg/mL.

Table AE-5: Raw data used for the detection of mRNA expression of key targets of fatty acid oxidation and synthesis from rat liver tissue cDNA samples by qPCR

Gene	OR			OP			T1C		
	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3
AMPK	19.82	21.94	20.13	21.45	23.32	19.58	20.73	22.67	19.26
	20.06	22.35	20.15	20.64	22.72	19.56	21.44	23.45	19.55
	19.23	21.18	20.33	20.92	23.18	19.90	21.38	23.53	19.97
	19.14	21.20	20.25	21.54	23.58	19.98	20.24	22.28	20.24
	19.64	21.57	21.02	19.97	22.11	20.13	20.75	22.68	21.16
	-	20.87	20.57	20.66	22.59	20.04	-	23.97	21.17
ACC	22.26	24.31	22.80	15.26	22.77	19.46	11.53	25.43	19.43
	22.96	36.78	23.15	23.29	23.29	19.04	30.55	26.27	19.37
	30.86	23.82	23.81	17.38	20.56	19.26	-	24.27	19.43
	-	22.24	20.97	19.04	22.47	19.09	26.60	24.08	19.14
	22.6	23.49	19.46	30.46	23.76	19.27	21.40	22.84	19.28
	22.63	18.58	24.23	12.15	30.40	19.30	13.85	28.57	19.23
CPT1	20.67	33.74	30.36	36.23	24.92	19.53	29.26	25.16	30.86
	28.51	34.80	35.41	36.32	26.78	21.39	35.01	14.63	39.98
	29.28	33.16	28.73	30.65	27.56	32.92	-	17.07	27.83
	38.25	30.67	14.33	17.68	24.16	26.67	33.49	29.93	30.36
	-	37.52	29.31	33.68	26.02	36.44	30.27	16.54	35.41
	30.78	33.52	28.97	16.27	13.65	26.50	13.62	20.44	28.73
FAS	33.22	31.77	26.06	30.67	32.34	27.25	30.78	26.06	21.92
	30.27	33.75	32.76	26.76	27.73	25.47	32.08	32.76	20.16
	34.80	28.54	33.46	30.54	29.03	24.61	33.54	33.46	20.60
	33.98	30.86	22.88	34.47	28.36	24.56	36.86	22.88	20.59

	30.82	32.99	36.63	25.68	29.21	25.11	34.27	36.63	20.62
	31.49	31.72	32.76	29.58	27.63	20.34	31.81	32.76	21.83
β actin	14.31	13.75	14.80	14.45	14.66	15.38	14.38	14.57	15.87
	14.4	14.43	15.22	14.33	14.27	15.12	14.41	14.34	15.51
	13.71	14.42	14.28	14.31	14.34	15.41	14.26	14.3	15.59
	14.38	14.17	14.52	14.31	14.66	15.45	14.45	14.22	15.02
	14.41	14.61	15.23	14.4	14.15	15.16	14.33	14.52	15.24
	14.26	13.82	15.22	13.71	13.6	14.58	14.31	14.56	15.52

Table AE-6: Raw data used for detection of mRNA expression of key targets of fatty acid oxidation and synthesis from rat muscle tissue cDNA samples by qPCR

Gene	OR		OP		T1C	
	Run 1	Run 2	Run 1	Run 2	Run 1	Run 2
AMPK	19.53	21.22	25.42	27.36	22.36	24.51
	24.77	26.93	23.99	25.83	25.11	27.57
	23.23	27.47	24.04	25.99	25.01	27.17
	28.06	30.14	27.04	29.07	-	36.67
ACC	23.15	23.82	36.38	33.93	20.13	19.43
	23.81	22.24	14.79	36.61	20.04	19.14
	20.97	24.31	22.80	23.68	21.38	19.28
	19.46	23.49	18.48	34.38	20.24	19.23
CPT1	20.92	19.40	23.29	25.53	20.92	22.11
	21.54	-	20.56	18.04	21.54	22.59
	19.97	21.18	22.47	24.12	19.97	22.77
	20.66	20.93	23.76	32.07	20.66	23.29
FAS	36.15	36.18	18.8	24.61	28.99	38.36
	32.43	-	21.92	24.56	35.02	38.78
	34.79	38.85	20.16	25.11	35.07	36.13
	30.10	38.86	20.60	20.34	31.80	36.15
β actin	14.8	14.28	15.38	14.27	15.59	15.24
	15.22	14.52	15.12	14.34	15.02	14.45
	14.28	15.23	15.41	14.66	15.24	14.33
	14.52	15.22	15.45	14.15	15.52	15.41

Table AE-7: Raw data used for detection of mRNA expression of key targets of fatty acid oxidation and synthesis from HepG2 cell cDNA samples by qPCR

Concentration	AMPK			ACC			CPT		
	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3
0	19.11	18.91	17.68	25.13	23.53	25.38	19.29	19.32	20.83
25	18.90	19.52	16.90	25.32	25.38	28.17	19.23	19.36	21.57
50	18.94	18.87	17.70	25.22	23.62	26.40	19.25	19.53	21.41
100	19.51	18.92	18.11	25.93	23.40	26.46	19.78	19.74	21.55

Concentration	FAS			β actin		
	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3
0	22.19	22.24	20.57	13.79	13.47	13.64
25	22.14	22.42	20.90	13.65	14.28	13.28
50	22.20	22.23	20.92	13.76	13.99	13.47
100	22.90	23.00	21.15	14.00	14.24	14.66

0, 25, 50, and 100 represent the concentrations of the LWDH ethanol extract in µg/mL.

Table AE-8: Raw data used for detection of mRNA expression of key targets of fatty acid oxidation and synthesis from L6 myotube cDNA samples by qPCR

Concentration	AMPK			ACC			CPT1		
	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3
0	17.49	16.12	15.49	15.82	12.5	12.33	17.02	17.86	15.09
FA	20.38	15.87	16.63	18.69	15.19	13.17	18.81	19.25	-
50	17.85	16.67	15.59	15.82	11.89	12.97	17.02	17.86	15.33
100	18.8	16.24	16.69	16.96	14.84	12.38	19.04	16.44	15.65
200	20.14	16.16	15.13	16.16	15.19	11.70	18.06	16.48	16.41

Concentration	FAS			β actin		
	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3
0	17.38	14.84	16.31	11.91	10.21	11.29
FA	17.11	16.31	14.43	10.28	11.52	10.85
50	17	16.65	15.87	11.36	10.02	12.23
100	15.97	18.46	15.59	11.54	11.47	11.64
200	15.78	17.32	16.75	10.64	9.87	10.83

0, 50, 100, and 200 represent LWDH ethanol extract concentrations in μg/mL. FA: fatty acid (palmitate; 0.25 mM/L).

APPENDIX F: BICINCHONINIC ACID ASSAY STANDARD CURVES FOR THE DETERMINATION OF TOTAL PROTEIN CONCENTRATION THROUGHOUT THE THESIS

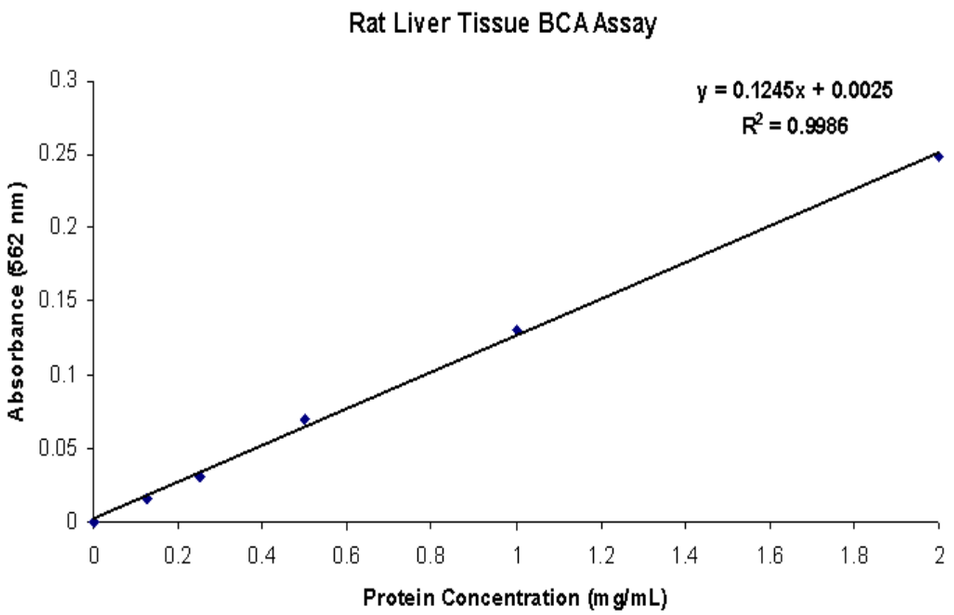

Figure AF-1: BCA assay standard curve for the determination of total protein concentration in rat liver.

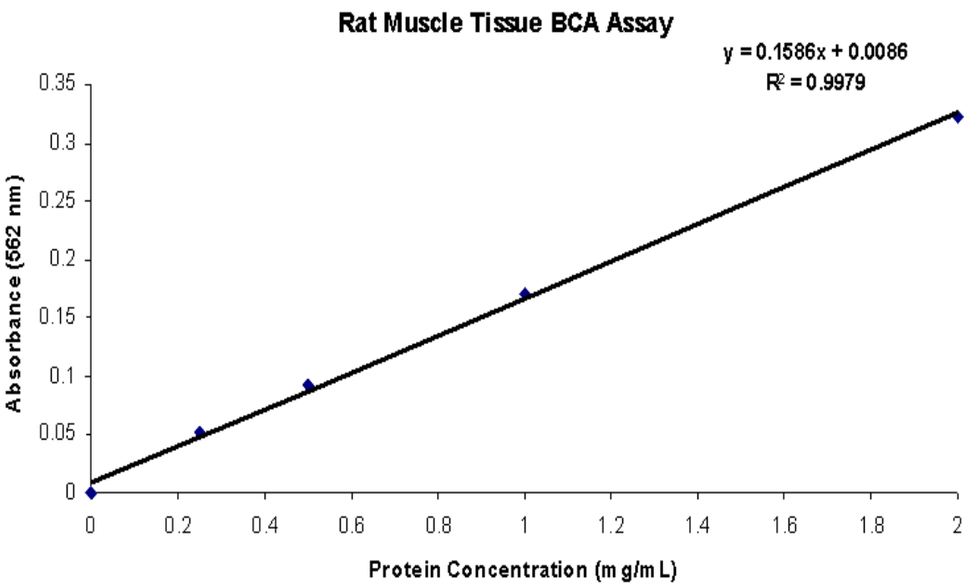

Figure AF-2: BCA assay standard curve for the determination of total protein concentration in rat muscle.

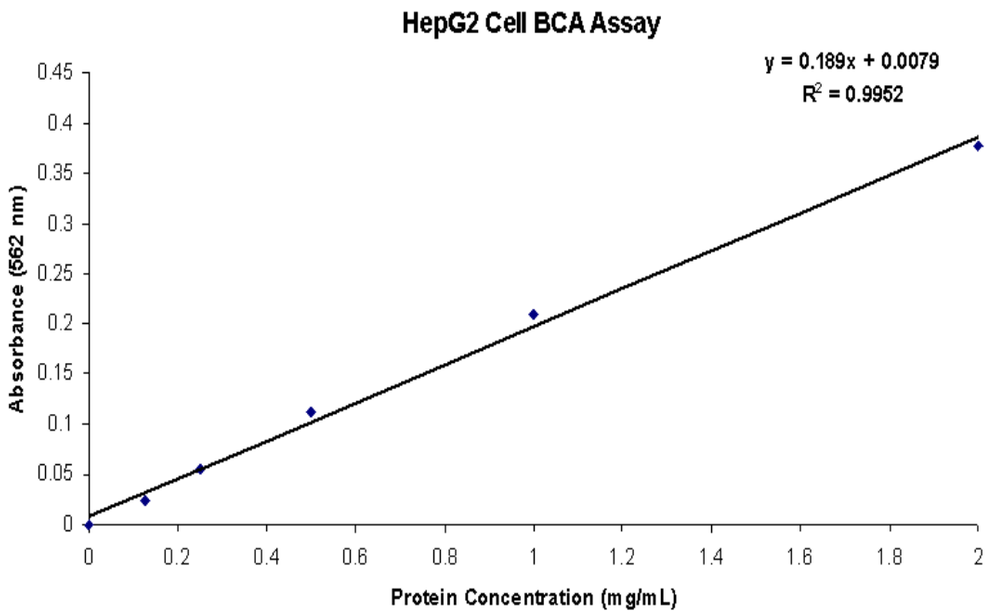

Figure AF-3: BCA assay standard curve for the determination of total protein concentration in HepG2 cells.

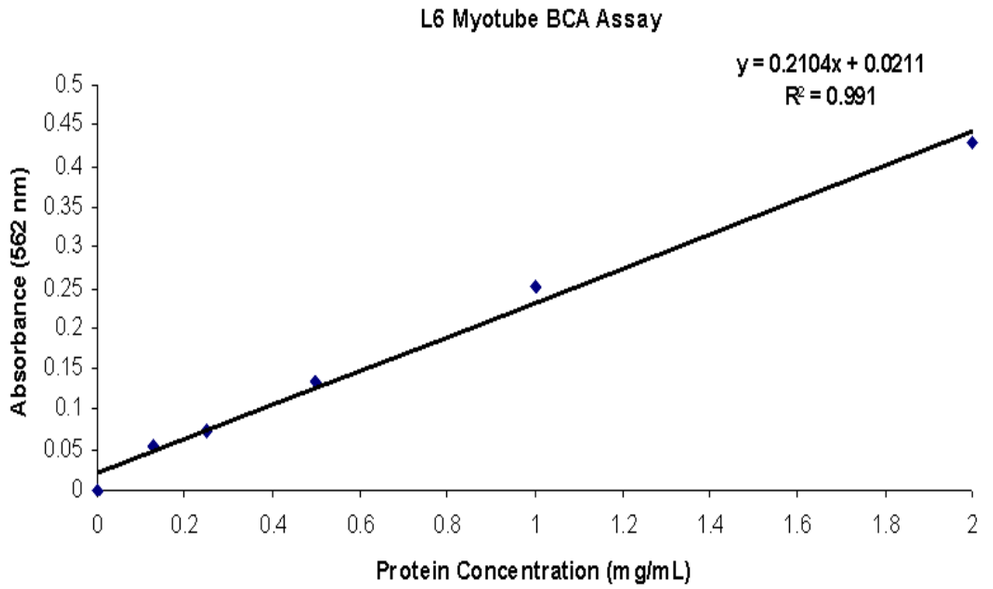

Figure AF-4: BCA assay standard curve for the determination of total protein concentration in L6 myotubes.

APPENDIX G: WESTERN BLOT RESOLVING AND STACKING GEL INGREDIENTS USED THROUGHOUT THE THESIS

Table AG-1: Western blot (a) resolving gel and (b) stacking gel ingredients[†*]

(a) Ingredient (μL)	7%	10%
30% acrylamide	2300	3300
4 X resolving gel buffer	2500	2500
dH_2O	5200	4200
25% AP	30	30
TEMED	5	5

(b) Ingredient (μL)	3%	4%
30% acrylamide	500	650
4 X stacking gel buffer	1250	1250
dH_2O	3300	3100
25% AP	15	15
TEMED	10	10

Resolving gel buffer (4 X): 9.09 g Tris base, 2 mL 10% sodium dodecyl sulfate (SDS), and enough dH_2O to reach a final volume of 50 mL (adjusted to pH 8.8). AP: ammonium persulfate dissolved to 25% using dH_2O; TEMED: tetramethylethylenediamine; stacking gel buffer (4 X): 3.03 g Tris base, 2 mL SDS, and enough water to reach a final volume of 50 mL (adjusted to pH 6.8). [†]Proteins > 100 kDa in size were run on gels comprised of 10 and 4% resolving and stacking gels, respectively. *Volume per gel.

APPENDIX H: DILUTION FACTORS AND HOST SOURCES OF ANTIBODIES USED FOR PROTEIN DETECTION THROUGHOUT THE THESIS

Table AH-1: Dilution factors and sources of primary and secondary antibodies used for detecting fatty acid oxidation and synthesis target proteins in rat liver and muscle tissues

Protein	1º Ab	Source	2º Ab	Source
AMPK	1:2500	Rabbit	1:5000	Anti-rabbit
pAMPK	1:1000	Rabbit	1:2000	Anti-rabbit
ACC	1:1000	Goat	1:2000	Anti-goat
pACC	1:1000	Rabbit	1:2000	Anti-rabbit
CPT1	1:500	Rabbit	1:1000	Anti-rabbit
FAS	1:1000	Rabbit	1:2000	Anti-rabbit
actin	1:2000	Rabbit	1:5000	Anti-rabbit

Antibody vendors and catalogue numbers: AMPK (Santa Cruz; sc-25792), pAMPK (Santa Cruz; sc-33524), ACC (Santa Cruz; sc-26822), pACC (Santa Cruz; sc-30446-R), CPT1 (Santa Cruz ; sc-98834), FAS (Novus Biologicals; NB400-14), actin (Santa Cruz, sc-1616), anti-rabbit 2º Ab (Santa Cruz goat anti-rabbit IgG-HRP, sc-2004), anti-goat 2º Ab (Santa Cruz donkey anti-goat IgG HRP, sc-2020).

Table AH-2: Dilution factors and sources of primary and secondary antibodies used for detecting fatty acid oxidation and synthesis target proteins in HepG2 cells

Protein	1º Ab	Source	2º Ab	Source
AMPK	1:2000	Rabbit	1:5000	Anti-rabbit
pAMPK	1:5000	Rabbit	1:2000	Anti-rabbit
ACC	1:500	Goat	1:1000	Anti-goat
pACC	1:500	Rabbit	1:1000	Anti-rabbit
CPT1	1:500	Rabbit	1:2000	Anti-rabbit
FAS	1:500	Rabbit	1:2000	Anti-rabbit
actin	1:2000	Rabbit	1:5000	Anti-rabbit

Antibody vendors and catalogue numbers: AMPK (Santa Cruz; sc-25792), pAMPK (Santa Cruz; sc-33524), ACC (Santa Cruz; sc-26822), pACC (Santa Cruz; sc-30446-R), CPT1 (Santa Cruz ; sc-98834), FAS (Novus Biologicals; NB400-14), actin (Santa Cruz, sc-1616), anti-rabbit 2º Ab (Santa Cruz goat anti-rabbit IgG-HRP, sc-2004), anti-goat 2º Ab (Santa Cruz donkey anti-goat IgG HRP, sc-2020).

Table AH-3: Dilution factors and sources of primary and secondary antibodies used for detecting fatty acid oxidation and synthesis target proteins in L6 myotubes

Protein	1º Ab	Source	2º Ab	Source
AMPK	1:2500	Rabbit	1:5000	Anti-rabbit
pAMPK	1:1000	Rabbit	1:2000	Anti-rabbit
ACC	1:1000	Goat	1:2000	Anti-goat
pACC	1:1000	Rabbit	1:2000	Anti-rabbit
CPT1	1:500	Rabbit	1:2000	Anti-rabbit
FAS	1:2000	Rabbit	1:5000	Anti-rabbit
actin	1:2000	Rabbit	1:5000	Anti-rabbit

Antibody vendors and catalogue numbers: AMPK (Santa Cruz; sc-25792), pAMPK (Santa Cruz; sc-33524), ACC (Santa Cruz; sc-26822), pACC (Santa Cruz; sc-30446-R), CPT1 (Santa Cruz ; sc-98834), FAS (Novus Biologicals; NB400-14), actin (Santa Cruz, sc-1616), anti-rabbit 2º Ab (Santa Cruz goat anti-rabbit IgG-HRP, sc-2004), anti-goat 2º Ab (Santa Cruz donkey anti-goat IgG HRP, sc-2020).